

APPLICATION OF FLUOROMETRY TO THE ANALYTICAL PROBLEMS CONCERNING
MARIJUANA IN BIOLOGICAL MATERIALS

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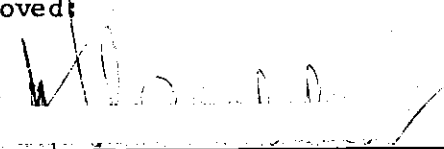
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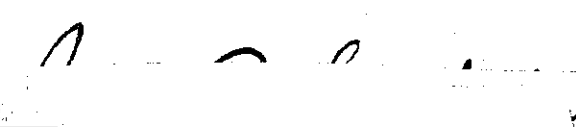
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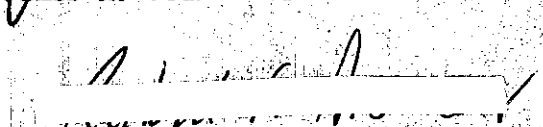
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Approved:


H.A. Flaschka, Chairman


James A. Stanfield


Richard F. Browner

Date approved by Chairman May 19, 1978

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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	iv
LIST OF ILLUSTRATIONS	v
SYMBOLS	vi
SUMMARY	vii
CHAPTER	
I. INTRODUCTION	1
II. INSTRUMENTATION, EQUIPMENT, AND CHEMICALS	10
III. EXPERIMENTAL	12
Hash Oil	
Stability of the Cannabinoids in Solution	
Spectrophotometry	
Extraction Procedures	
Derivatives of Tetrahydrocannabinols	
IV. RESULTS AND DISCUSSION	25
Stability	
Spectrophotometry	
Extraction Procedures	
Derivatives of Tetrahydrocannabinols	
CONCLUSIONS	43
APPENDIX A	44
APPENDIX B	54
APPENDIX C	56
BIBLIOGRAPHY	57

LIST OF TABLES

TABLE	Page
1. Tabulation of Experimental Conditions for the von Pechmann-Duisberg Reaction	21

LIST OF ILLUSTRATIONS

FIGURE		Page
1.	Cannabinoidic Numbering Systems	4
2.	Apparatus for Low Temperature Experiments	17
3.	Stability Data	26
4.	Fluorescence Spectrum of THC	28
5.	Decomposition of Δ^9 -THC Upon Irradiation	31
6.	Temperature Dependence in CO ₂ /Acetone Coolant	32
7.	Temperature Dependence in Liquid Nitrogen Coolant	33
8.	Temperature and Time Dependence in Sulfuric Acid/THC Reaction	42
9.	Spectrophotometric Data for Δ^8 -Tetrahydrocannabinol	45
10.	Spectrophotometric Data for Δ^9 -Tetrahydrocannabinol	46
11.	Spectrophotometric Data for Cannabidiol	47
12.	Spectrophotometric Data for Cannabinol	48
13.	Spectrophotometric Data for Cannabichromene	49
14.	Spectrophotometric Data for Cannabicyclol	50
15.	Spectrophotometric Data for Cannabigerol	51
16.	Infrared and Ultraviolet Spectra for Compound Labeled "Top Band"	52
17.	Infrared and Ultraviolet Spectra for Compound Labeled "Bottom Band"	53

SYMBOLS

A	Absorbance
c	Concentration, grams/liter
d	Optical depth of solution
ϵ	Molar absorptivity, liter/mole-centimeter
E1%	Intensity of absorption (the absorbance of a 1% solution in a 1.0 cm cell)
F	Fluorescence intensity (area under the fluorescence spectrum)
nm	Nanometer
%T	Percentage transmittance
μl	Microliters, 10^{-6} liters
λ_{max}	Wavelength of an absorption maximum
ϕ	Quantum efficiency

SUMMARY

The object of this research was to find a simple and rapid method of analysis to detect cannabis intoxication by the application of fluorometry.

The native fluorescent properties of some of the cannabinoids were studied. With regard to tetrahydrocannabinols, data were collected concerning the dependence of the fluorescence intensity on temperature and pH value. The effects of decomposition during irradiation were measured and the quantum efficiency and molar absorptivity were calculated. The stability of the cannabinoids in organic solvents was also studied. An extraction method was evaluated for its effectiveness in removing the cannabinoids from biological fluids.

Highly fluorescent compounds were obtained by using a procedure based on the von Pechmann-Duisberg synthesis of coumarins and by using a procedure based on the Dal Cortivo method of dimer formation.

Infrared, nuclear magnetic resonance, mass spectral, ultraviolet, and fluorescence data have been recorded for the compounds prepared by the von Pechmann-Duisberg procedure.

CHAPTER I

INTRODUCTION

Indian Hemp, Cannabis sativa L., is a common weed which grows in all parts of the world. It is classified as a dioecious plant that may attain heights of 16 feet or more and is used as a medicine, an intoxicant, and a source of fiber. It is more commonly called marijuana and this term applies to the drug as well as to the plant. Marijuana has been known as a psychoactive agent for over 3000 years and by 500 B.C. was apparently in wide use in Central Asia, India, and China. It ranks along with alcohol, caffeine, and tobacco as one of the most widely used drugs. The psychoactive ingredients of the plant are introduced into the blood system either by smoking or by ingesting the dried leaves. It is estimated that throughout the world there are over 300 million regular marijuana users (1). However, in the United States the use of the cannabis plant as a drug was on a very low level until the late 1950's. Since then, the problem of cannabis consumption has expanded dramatically.

With the vast increase in cannabis usage, there is growing concern for the effects of marijuana on its users, in particular for harmful side effects. In small dosages, marijuana produces a mild euphoria while with larger dosages hallucinogenic activity can occur. Marijuana can cause changes in personality, memory, facial expressions, mood, motivation, skin color, and motor coordination. Thus, driving an automobile or operating machinery while under the influence of marijuana

creates a potentially dangerous situation. While drunk driving is still the greatest cause of traffic accidents, driving under the influence of cannabis is increasing at an alarming rate. It is conceivable that in the near future the problem of cannabis intoxication will replace alcohol intoxication as the most single important cause in traffic deaths. This will be especially the case if marijuana is decriminalized or legalized throughout the United States.

Studies have shown that more cannabis users appear to drive today while intoxicated than was the case a few years ago. In one such survey (2), up to 80 percent of the users questioned reported that they had driven soon after using marijuana. In another study (3) of 267 traffic deaths in which drug use could be verified as a potential cause, the researchers found that 31 percent of the accidents could be directly attributed to marijuana. Of special significance is the fact that 64 percent of the drivers were either regular smokers of marijuana or had experimented with the drug at some time.

Even after marijuana use is completely abandoned, it may take months for all the active components, the tetrahydrocannabinols (commonly referred to as THC) and their metabolites, to be excreted from the body. The tetrahydrocannabinols are insoluble in water but are highly soluble in fat. This means that with continued use the drug will accumulate in the fat structures of the cells. Some of the THC absorbed by the body is gradually metabolized into 11-hydroxy-tetrahydrocannabinol which is a substance believed to have even greater psychoactive effects than the parent compounds. Thus the marijuana user can be under the influence of the drug even between peak "highs". Since the persistent effects of the

drug are known to include impaired judgment, lower attention span, poor perception of time, distance, and speed, and defective motor coordination, it seems likely that the driving ability of a chronic marijuana user will be severely hampered.

The increasing use of alcohol and marijuana taken together poses an even greater threat to traffic safety. One recent survey (2) reported that 64 percent of those persons questioned had used a combination of alcohol and marijuana before driving. It has been demonstrated that two drinks of alcohol plus a moderate dose of marijuana will cause heavy intoxication. Thus it becomes important to detect cannabis intoxication with simple and rapid methods of analysis analogous to those already existing for alcohol.

A survey of the literature reveals that research involving marijuana has been going on since the turn of the century; however it was not until 1964 that the tetrahydrocannabinols were isolated in pure form. At the present time, one can identify about fifty potentially active compounds in the cannabis plant, including thirty-seven cannabinoids and a number of alkaloid-type substances. But only two isomers of the tetrahydrocannabinol are reported to cause euphoria. Some confusion is caused by the fact that two different numbering systems are being used when naming these two isomers. In most American publications a formal numbering system is used which is based on the pyran ring. The isomers are written as Δ^8 -tetrahydrocannabinol and Δ^9 -tetrahydrocannabinol (Figure 1a). In European publications a numbering system is used which is based on the monoterpenoid moiety. Thus the two isomers are called Δ^1 -tetrahydrocannabinol and Δ^6 -tetrahydrocannabinol (Figure 1b). This

paper will use the numbering system based on the pyran ring.

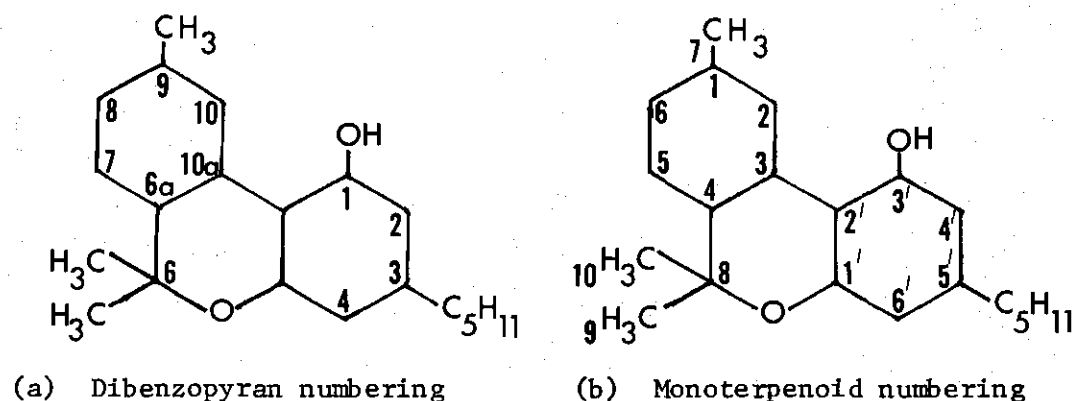


FIGURE 1. Cannabinoidic Numbering Systems

The development of sufficiently sensitive and selective analytical techniques has made the identification and, in some cases, even the quantitation of these components relatively simple. In fact, scientists engaged in the field of forensic drug identification analyze materials containing cannabis more frequently than all other legal or illicit drugs combined. However, the identification and especially the determination of cannabinoids in biological samples is still a major problem.

Numerous papers have been published on various methods to determine cannabis in biological fluids. Most of these are not suitable for quick, accurate but inexpensive marijuana determinations by a clinical or forensic laboratory. Much of the literature deals with procedures that are either too costly to implement or are not reliable enough to use in legal proceedings, or both. Studies have been shown that an analytical method capable of detecting 2 ng/ml of tetrahydrocannabinol in blood is required. At this low level compounds in both urine and

blood that can interfere with an analysis are very numerous. The problem is complicated by the fact that these compounds can differ from person to person and for a given person from time to time. Removal of the interferences is often necessary and this can be very costly in terms of time, materials, and equipment.

Any extraction procedure used to isolate the cannabinoids from the biological fluids must take into account the significant solubility of the cannabinoids in lipids. Both these problems due to interferences and to lipid solubility must be solved before an analytical method can be successful.

Over the past few years many techniques have been utilized, some with limited success. As early as 1967, da Silva (4) claimed that, by using thin layer chromatography (TLC), he detected some of the cannabinoids in blood, urine, and saliva from people intoxicated with cannabis. However, subsequent workers have failed to confirm most of his results. Although Just (5) and Forrest (6) have proposed improvements in the selectivity and sensitivity for the application of thin layer chromatography, the poor resolution of the large number of cannabinoids and other compounds present in the sample is still a severe limiting factor.

Gas-liquid chromatography (GLC) would seem to be the method of choice for rapid analysis. A large variety of stationary phases have been found to separate the cannabinoids on packed columns. GLC also provides a wide variety of detection methods. Electron capture detection of cannabinoids based on the derivatization of the phenol with halogen-containing reagents has been developed. Fenimore (7) utilized

heptafluorobutyric acid as a derivatizing reagent and claimed levels of 0.1 ng/ml in plasma were obtainable while Garrett (8) used pentafluorobenzoic acid to detect levels of 125 pg/ml in plasma. Since these detectors respond to all strongly electron-capturing compounds plus any other compounds having hydroxy or amino groups that are capable of reacting with the derivatizing agents used, the selectivity of the procedures is very low. Furthermore, electron capture detectors usually produce a noisy background. To increase the signal to noise ratio, McCallum (9) developed a procedure using the flame ionization detection of the phosphate ester derivative of Δ^9 -THC. The sensitivities of both the electron capture and the flame ionization methods are impressive, but the problem of interfering compounds still remains.

Gas chromatography/mass spectrometry (GC/MS) techniques have been utilized because of the capability of providing adequate sensitivity. Agurell (10) devised a preliminary purification of the extract from human plasma by chromatography on a Sephadex LH-20 column that gave adequate clean-up for subsequent quantification of the Δ^8 -THC by mass fragmentometry. Here, mass fragmentometry provided what was in effect a highly selective GLC detector. Agurell used a deuterated Δ^8 -THC internal standard and a computer system that simultaneously scanned four different masses. A similar technique was designed by Rosenfeld (11) who used Δ^9 -THC perdeuteriomethyl ether as an internal standard. One of the problems with these two methods is that the appropriate isotopically labeled standard must be synthesized. Another problem is the possible contamination of the sample by endogenous compounds. The purification on Sephadex LH-20 can result in a 30 percent loss of the cannabinoids (12).

Further, the cost of using a GC/MS with a computer system capable of handling the complex programs is probably beyond most laboratories.

Several investigators are working with immunoassay methods but as yet none of these methods is applicable to the quantification of THC in human plasma. Cais (13) has developed a free-radical immunoassay procedure similar to the one available for morphine but the sensitivity does not seem to be high enough for the direct screening of urine samples.

There are other analytical techniques available to the chemist, among these, fluorometry. Fluorometry is one of the most sensitive analytical tools for the detection of a wide variety of compounds. But in the application to cannabis determination, surprisingly little work has been reported. The fluorescence and phosphorescence spectra of some of the cannabinoids in ethanol have been described by Bowd (14). Bowd's paper deals with the decomposition of the tetrahydrocannabinols and no attempts were reported on applying fluorometry to detect THC in biological fluids. Recently (5) it was proposed that the formation of 1-dimethylamine-naphthalene-5-sulfonyl chloride (dansyl-Cl) derivatives followed by fluorometry would be a possible means of detecting Δ^8 -THC in body fluids. Forrest (6) investigated this possibility by preparing fluorescent-labeled derivatives of several cannabinoids. The resultant mixture was separated by thin layer chromatography with a reported detectable level of 0.5 ng. The reaction yielded di-dansylated as well as mono-dansylated derivatives. Forrest's method has not been utilized because of the instability of the dansylated cannabinoids and the non-selectivity of the method. Vinson (15) synthesized a new fluorescent

derivative using 2-p-chloro-sulfophenyl-3-phenylindone (dis-C₂). He tested twenty-six common drugs and reported that they did not interfere with the detection of THC. Both of these methods were tested in our laboratory. The Forrest method did not give reproducible results while the dis-C₂ method by Vinson did not have the required sensitivity.

A method using a gallium chelate formation has been devised by Bourdon (16). This technique involves using an O-O'-dihydroxy-azo derivative of tetrahydrocannabinol that forms a highly fluorescent chelate with gallium. Any compounds present in the biological sample that react with the reagents will give the same signal, thus causing erroneous results. The relatively high limit of detection (1.0 µg) is also a disadvantage.

The inherent problem with the above mentioned methods is that the fluorescence of the tag is being measured. Instead of labeling the tetrahydrocannabinols with a reagent that has fluorescence, a better approach would be to produce a derivative, the fluorescence of which is dependent upon the parent cannabinoid rather than upon the derivatizing reagent. Bullock (17) had already attempted to use this approach. He proposed the detection of cannabis in biological fluids by condensing the cannabinoids with a polycarboxylic acid to give highly fluorescent γ-lactone cannabinoids. By maximizing the intensity of the fluorescence in the pH range of 9-11, he found the detection of as little as 0.6 mg of marijuana to be realizable. It should be possible to find other reactions that are similar to those employed by Bullock.

It was hoped that deliberate application of fluorometry to the analytical problems concerning marijuana in biological material would have some chance of success; that is, of providing the possibility for

a fast, inexpensive, and accurate determination. Unfortunately as the investigations progressed, the problems and difficulties accumulated. The lack of adequate fluorescent specificity in the nanogram range as well as the necessity for highly complex, expensive equipment were the chief causes for the failure to achieve the hoped for goals. However, the research did produce some interesting findings that have scientific merit and are worthy of reporting.

An extraction method was evaluated for its effectiveness to remove the cannabinoids from biological fluids. Since in man some of the cannabinoids are bound to proteins in the blood, the extraction method has to be able to remove both the free drug, the protein-bound drug, and the drug stored in the fat cells. It was found that a mixture of isoamyl alcohol and heptane yields an extraction efficiency of up to 98% and higher. The native fluorescent properties of some of the cannabinoids were studied. With regard to tetrahydrocannabinols, data were collected concerning the fluorescence intensity dependence on temperature and pH value. The effects of decomposition during irradiation were measured. The quantum efficiency and molar absorptivity for the tetrahydrocannabinols were calculated. The stability of the cannabinoids in organic solvents was also studied.

Highly fluorescent compounds can be synthesized by condensation of phenols with β -keto esters or similar compounds in the presence of condensation agents such as sulfuric acid, phosphorus pentoxide, or phosphorus oxychloride. These procedures are based on the von Pechmann-Duisberg synthesis of coumarins. Part of the research dealt with the formation of intensely fluorescent compounds by the reaction of tetrahydrocannabinol with ethyl acetoacetate.

CHAPTER II

INSTRUMENTATION, EQUIPMENT, AND CHEMICALS

Instruments

The fluorescence excitation and emission spectra were obtained with a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer using Fisher-brand Suprasil fluorescence cells. To the instrument was attached a constant temperature accessory for the low temperature experiments. A flow-through liquid chromatograph cell was used to interface the fluorometer to a Varian 8500 high pressure liquid chromatograph.

The absorption spectra were obtained with a Beckman ACTA CIII ultraviolet spectrophotometer using Fisher-brand Suprasil ultraviolet cells.

The infrared spectra were measured with a Beckman 4240 infrared spectrophotometer using standard NaCl salt plates. Mass spectra and nuclear magnetic resonance spectra were recorded with a Finnigan 3100D gas chromatograph/mass spectrometer and a Varian T-60A NMR spectrometer with a Nicolet 1020A signal averaging system, respectively.

All gas chromatograms were obtained with a Hewlett-Packard 5830A gas chromatograph using a 3% OV-1 80-100 mesh chromosorb W.H.P. column. A standard 5 μ l Hamilton syringe was employed.

Precoated thin layer chromatography plates, Silica Gel G., 250 microns by Analtech, were used in standard rectangular thin layer tanks.

All weighings were done on a Mettler H-18 analytical balance.

Glassware

The usual glassware such as beakers, flasks, separatory funnels, etc., were used as needed. In addition, disposable micropipettes by Yankee in 1,2,3,4,5 and 10 μ l denominations and Applied Science Laboratories 3-ml reaction mini-vials were utilized. A micro distillation apparatus was used for purification of some of the reagents.

Chemicals

The sulfuric acid was MCB fluorometric grade reagent. All solvents were of spectroanalytical grade. All other reagents were of analytical grade.

Some of the cannabinoids were purchased from two chemical supply companies. Δ^9 -tetrahydrocannabinol and Δ^8 -tetrahydrocannabinol as authentic substances were obtained from U.S.P.C. The following NIDA standards were purchased from Applied Science Laboratories:

Cannabinol, NDC 00079-020503-26004
Cannabichromene, NDC 00079-0201-03-26001
Cannabigerol, NDC 0079-0204-03026003
Cannabicyclol, NDC 00079-0202-03-26002
Cannabidiol, NDC 00079-0203-03-26032
 Δ^9 -tetrahydrocannabinol, NDC 00079-0229-03-26028
 Δ^8 -tetrahydrocannabinol, NDC 00079-0228-03-26027

Large quantities of Δ^9 -THC in the form of hash oil were obtained from Atlanta Police Department drug cases. Hash oil is an oily, thick, organic extract of the marijuana plant. It can contain up to approximately 40% Δ^9 -THC. The Δ^9 -THC was extracted from the hash oil using a Varian 8500 high pressure liquid chromatograph with a Partisil 20 column.

CHAPTER III

EXPERIMENTAL

The ultraviolet, fluorescence, and infrared spectrophotometers, and the nuclear magnetic resonance and mass spectrometers were calibrated before any data were recorded. All of the quartz cells and reaction mini-vials were washed with a soap solution, rinsed with distilled water, and then rinsed with absolute ethanol and dried before use. The disposable pipettes were used only once. When possible, all reagents and chemicals were stored in their original containers to prevent contamination from plasticizers. No plastic products came into contact with any solvents or solutions.

Hash Oil

Some of the Δ^8 -tetrahydrocannabinol and Δ^9 -tetrahydrocannabinol that were purchased from the chemical supply companies arrived in the laboratory as unusable, degraded samples. Instead of purchasing from these expensive commercial sources, large quantities of Δ^9 -THC were obtained from extractions of hash oil, a thick, oily organic extract of the marijuana plant. The material containing Δ^9 -THC was obtained by dissolving 1 gm of oil in 100 ml of anhydrous ethyl ether. The solution was decolorized by adding approximately 5 gm of activated carbon while stirring, then filtered to remove the activated carbon, and finally evaporated to dryness. The residue was dissolved in 5 ml of cyclohexane:dioxane (9:1). By using a high pressure liquid chromatograph

with a Partisil 20 column at a flow rate of 60 ml/hr and employing a refractive index detector, the Δ^9 -THC fraction emerging at 6 minutes was collected. This fraction was evaporated to dryness using low heat and a stream of nitrogen, and then redissolved in absolute ethanol. The purity of the 63 mg of sample collected was determined by gas chromatography to be 96.5%. This procedure was repeated whenever Δ^9 -THC was required for the derivative experiments described later in this chapter.

Stability of the Cannabinoids in Solution

For the purposes of this research, it was necessary to store dilute solutions of THC. Ethanol was the solvent of choice since other solvents were unsuitable as a vehicle for delivery into blood and water samples. However, it was discovered that solutions with these low concentrations of THC were unstable unless special precautions were taken. The two main problems that developed were (i) Δ^9 -THC was easily oxidized to cannabinol and (ii) Δ^8 -THC and Δ^9 -THC formed as yet unidentified polymeric compounds. The major sources of these problems were attributed to the influence of light and oxygen. The problem of light was solved by using amber glassware and by storing the solutions in darkness. For solving the problem of oxygen, experiments were devised to determine what, if any, additives or special handling techniques could eliminate or at least reduce the oxidative decomposition.

The first step taken was to have all of the inside of the amber volumetric flask silanized using a solution of toluene and dimethyldichlorosilane (DMCS). The flask was completely filled with a solution of 10% DMCS in toluene for several minutes, emptied, and then allowed to

air dry. Next, five solutions of Δ^9 -THC were prepared. The first solution was made by dissolving 800 μg of THC in 10 ml of absolute ethanol. The other four solutions were similarly prepared but using a specially treated ethanol. The alcohol was treated to remove most of the dissolved oxygen by using a nitrogen gas purge. However, it was first necessary to purify the nitrogen by passing it through a solution containing amalgamated zinc and ammonium metavanadate in order to remove the small amounts of oxygen present in the nitrogen. This purified nitrogen was then bubbled through the ethanol. To the second, third, and fourth solutions, NaNO_2 , TiCl_3 , and ascorbic acid were added, respectively. Nothing was added to the fifth solution. Ultraviolet and fluorescence scans were recorded every day for a period of several weeks. Between the scans, the five solutions were purged with nitrogen, sealed, and stored at -20°C .

Spectrophotometry

The absorption spectra of each solution containing 100 $\mu\text{g}/\text{ml}$ of NIDA standard were obtained with the ultraviolet spectrophotometer. Approximately 3 ml of standard solution was placed in one of a matched pair of Fisher-brand Suprasil ultraviolet cells and 3 ml of absolute ethanol was placed in the other cell as a reference. The standard solutions were scanned from 220 nm to 340 nm at a %T mode.

The native fluorescence spectra were measured by placing approximately 3 ml of each standard solution in Fisher-brand fluorescence cells and then scanning both the emission and excitation wavelengths in the fluorescence spectrophotometer. The initial excitation wavelength was determined by calculating the absorbance maximum of the spectrum. The

optimal fluorescence spectra were obtained by appropriately adjusting the emission and excitation slits.

Additional solutions were prepared by replacing ethanol with methanol, hexane, heptane, and isoamyl alcohol, respectively. The fluorescence spectrum of each of these solutions was determined in an analogous manner. Blank solutions of each solvent were measured as a reference to ensure that no fluorescent impurities were present. Additional standard solutions in concentrations of 100, 80, 40, and 2 ng/ml of Δ^8 -THC in ethanol were prepared and their spectra recorded in order to find the limits of detection by fluorometry.

The quantum efficiency of Δ^8 -THC was calculated by using the following procedure. The emission spectra of a standard solution of 100 $\mu\text{g/ml}$ of Δ^8 -THC in ethanol and of a solution of 0.1 N quinine in 0.1 N sulfuric acid were obtained with all instrumental settings identical except that of the excitation monochromator. The Δ^8 -THC solution was excited at 282 nm while the quinine solution was excited at 350 nm. Each spectrum band was cut out and integration obtained by weighing on a Mettler analytical balance. The E1% of Δ^8 -THC was calculated by first making a 100% T correction at 282 nm with a blank ethanol solution and then measuring the %T of the 100 $\mu\text{g/ml}$ of Δ^8 -THC solution against a reference of absolute ethanol. The absorbance of the quinine solution was similarly measured except that an excitation wavelength of 350 nm was used. The data obtained along with the known quantum efficiency value for quinine were used to calculate the quantum efficiency of Δ^8 -THC by using the equation

$$\frac{F_2}{F_1} = \frac{\phi_2}{\phi_1} \cdot \frac{A_2}{A_1} \quad (I)$$

Details of the calculations can be found in the Appendix C.

The variations or differences in the fluorescence intensities in acidic and basic solutions were measured by the addition of one drop of concentrated hydrochloric acid and one drop of concentrated sodium hydroxide to cuvettes containing the THC standard solutions. The emission spectra of the acidic and the basic solutions were recorded and then compared with the emission spectra of the solutions before the addition of any acid or base.

The effects of prolonged irradiation on Δ^9 -THC were monitored over several days by using the ultraviolet source from the fluorometer. A standard Δ^9 -THC solution was placed in a quartz cuvette and the cuvette was sealed with a Teflon cap. The excitation wavelength was adjusted to 282 nm. Emission spectra were obtained every 24 hours for three days. All the instrumental parameters were kept constant during this time period.

The effects of temperature on the fluorescence of Δ^9 -THC were determined in three experiments. The cell compartment in the fluorometer was modified to accommodate a constant temperature cell provided by the Perkin-Elmer Corporation. A nitrogen purge was attached to the cell compartment in order to remove any water vapor and thus prevent condensation at low temperatures. In the first experiment, a closed pumping system composed of a water pump, a glass tank, flexible Teflon tubing, and coiled copper tubing was constructed such that cooled acetone could be pumped through the constant temperature cell block.

(Figure 2). The temperature in the quartz cell was measured with a

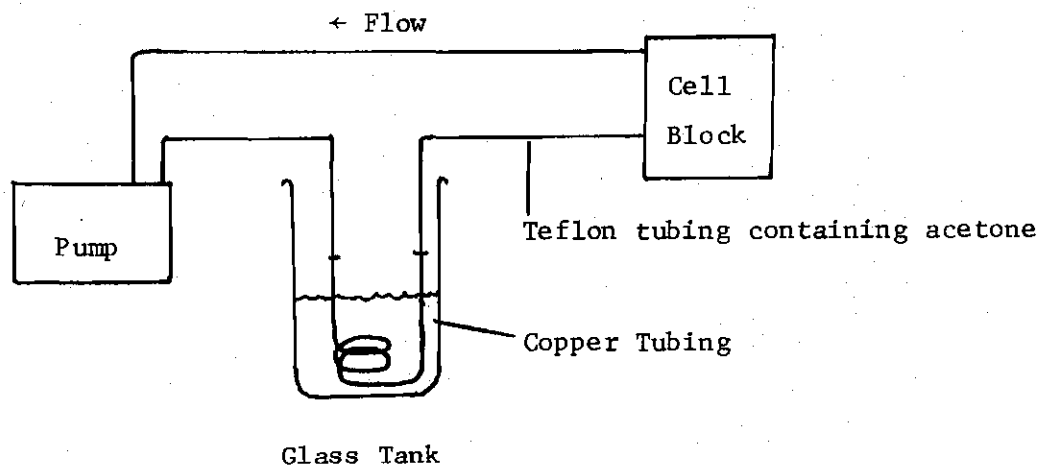


FIGURE 2. Apparatus for Low Temperature Experiments

-50°C to +50°C thermometer immersed in the solution so as not to obstruct the excitation beam. The acetone coolant was chilled with an acetone-ice-water mixture. The cell block was allowed to equilibrate for 15 minutes before temperature and emission measurements were recorded.

The same equipment was employed in the second experiment except that an acetone-dry ice mixture was used to cool the acetone in the pumping system. The cell was allowed to equilibrate for 15 minutes before the pump was stopped. After the initial temperature and emission spectra were recorded, the cell compartment temperature was allowed to rise to ambient temperatures. During this period of time, emission spectra were recorded at various temperatures.

In the third experiment, liquid nitrogen was used to freeze the standard solution to a solid. A cuvette containing the standard 100 $\mu\text{g/ml}$ Δ^9 -THC in ethanol solution was dipped into the liquid nitrogen for 5 minutes. The cuvette was then transferred under a stream of nitrogen to the cell compartment which in turn had been purged with nitrogen. An additional stream of nitrogen was directed onto the surface of the cuvette to prevent condensation of humidity on the quartz surfaces while the cuvette was in the cell compartment. The instrumental parameters were adjusted to the same conditions as the first two experiments. The fluorescence at 306 nm was recorded.

Extraction Procedures

The next step in the research was to find an extraction method capable of removing the cannabinoids from biological fluids with an adequately high efficiency. The n-heptane/isoamyl alcohol mixture recommended by Lemberger (18) seemed to offer the best chance. A series of extraction procedures first involving water solutions and then blood samples was devised in order to find the optimum conditions. First, the E1% of Δ^8 -THC in n-heptane was calculated by evaporating to dryness 2 ml of an ethanol solution containing 200 μg of Δ^8 -THC under low heat and under a stream of nitrogen. The residue was dissolved in 4 ml of n-heptane and the solution scanned on the spectrophotometer for the %T at 282 nm. Having determined the E1% of Δ^8 -THC in n-heptane, it was possible to calculate the extraction efficiencies achieved in the experiments.

The procedure that was found to have the highest efficiency was the one in which 5 ml of water solution or 5 ml of a blood sample, each containing 200 μg of Δ^8 -THC, was placed in a separatory funnel and extracted twice with 5-ml portions of a n-heptane/isoamyl alcohol (98.5:1.5) solution. The combined organic layers were washed with 10 ml of distilled water and then extracted twice with 5-ml portions of a modified 1/10 Claisen's alkali solution (3.5 g KOH dissolved in 25 ml water, and 100 ml of methanol added). The alkaline phases were combined, made slightly acidic by the addition of concentrated hydrochloric acid, diluted with distilled water to dissolve any solid potassium chloride formed, and finally extracted with 4 ml of n-heptane. The organic layer was transferred by pipette to a quartz cuvette and scanned on the ultra-violet spectrophotometer between the wavelengths of 220 nm and 340 nm.

Other experiments did not yield as high extraction efficiencies, e.g. changing the ratio of the n-heptane/isoamyl alcohol mixture from 98.5:1.5 to 85:5, or using one 10-ml portion of n-heptane/isoamyl alcohol mixture instead of two 5-ml portions. In all of these extraction experiments, 10 μl micropipettes were used to introduce the desired amounts of Δ^8 -THC into water or blood samples.

Derivatives of Tetrahydrocannabinols

Both Δ^8 -THC and Δ^9 -THC produce the same fluorescence spectrum near 306 nm when excited at 282 nm. This spectrum lies within a region where a large number of impurities also give fluorescence. Unfortunately, the presence of these fluorescent impurities becomes a significant factor when dealing with the 2 nm/ml levels of THC which may be present in

blood. It was considered, however, that if a fluorescent derivative of tetrahydrocannabinol could be synthesized so as to effect a bathochromic shift in the emission spectrum, many of the problems of contamination could be solved.

Highly fluorescent compounds can be synthesized by condensing phenols with β -keto esters or similar compounds. This synthesis is based on the von Pechmann-Duisberg formation of coumarins (19). Since tetrahydrocannabinol has a phenolic group, it was hoped that a fluorescent compound could be generated by reacting THC with ethyl acetoacetate in the presence of dehydrating agent. To test this procedure (20), a coumarin was synthesized by adding 1 ml of a 2% solution of ethyl acetoacetate in ethanol to 1.5 ml of a 10% solution of resorcinol in absolute ethanol. Concentrated sulfuric acid (2.5 ml) was then added to the mixture by letting the acid run down the walls of the vessel while stirring. The solution was allowed to stand at room temperature for 15 minutes before the emission wavelengths were scanned at an excitation wavelength of 366 nm. An intense fluorescence was observed at 430 nm.

The above procedure was repeated except for the fact that 1.0 ml of a solution of 10 mg/ml of Δ^9 -THC in absolute ethanol was substituted for the resorcinol solution. Neither a reaction nor new fluorescence were observed.

Various procedures were tried in which the concentrations of the sulfuric acid and the ethyl acetoacetate were varied. Different dehydrating agents such as phosphorus pentoxide and phosphorus oxychloride were substituted in place of sulfuric acid. By using a hot water bath and an autoclave, the temperature, pressures and times of the reactions were varied (Table 1).

TABLE 1. Tabulation of Experimental Conditions for the von Pechmann-Duisberg Reaction

To 1 ml of 100 $\mu\text{g/ml}$ of Δ^9 -THC in ethanol solution, the following reagents were added and the experimental conditions were varied.

Dehydrating Reagent	Ethyl Acetoacetate (in EtOH)	Temperature °C	Time (min)
3 drops H_2SO_4	1 ml 5%	255*	20
6 drops H_2SO_4	1 ml 5%	255*	20
15 drops H_2SO_4	1 ml 5%	255*	20
0.1 drop H_2SO_4	1 ml 5%	255*	20
10 drops H_2SO_4	1 ml 5%	255*	20
10 drops H_2SO_4	3 ml 5%	255*	20
10 drops H_2SO_4	1 ml 25%	255*	20
10 drops H_2SO_4	1 ml 100%	255*	20
10 drops H_2SO_4	1 ml 5%	70	20
10 drops H_2SO_4	1 ml 5%	90	20
3 drops H_2SO_4	1 ml 5%	260*	20
5 drops H_2SO_4	1 ml 25%	240**	20
3 drops H_2SO_4	1 ml 5%	RT	300
3 drops H_2SO_4	1 ml 5%	RT	1440
3 drops H_2SO_4	1 ml 5%	50	1440
2 ml P_2O_5 +	1 ml 5%	60	60
2 ml P_2O_5 +	1 ml 5%	255*	20
10 drops POCl_3	1 ml 5% ++	210*	10
10 drops POCl_3	1 ml 5% ++	RT	60
10 drops POCl_3	1 ml 5% ++	90	60
10 drops POCl_3	1 ml 5% ++	90	120
10 drops POCl_3	1 ml 5% ++	90	240

Notes: * Autoclaved (20 PSI)

** Autoclaved (12 PSI)

+ 100 mg P_2O_5 /20 ml EtOH

++ Benzene solution

All experiments were run in triplicate with blanks as reference.

The procedure used to produce the fluorescence compound involved pipetting 1 ml of a solution containing 100 $\mu\text{g/ml}$ of Δ^9 -THC in ethanol into a 3-ml reaction mini-vial. To this mini-vial, 1 ml of a freshly distilled 5% ethyl acetoacetate in ethanol solution was added. The vial containing the mixture was placed in an ice water bath, and when cooled to approximately 0°C , 10 drops of concentrated fluorometric grade sulfuric acid was slowly added to the mixture. The vial was sealed with a screw cap, shaken to mix the solution, unscrewed to release the pressure, and then tightly resealed. After submerging the vial in a hot water bath at 90°C , and allowing a reaction time of 90 minutes, the vial was transferred to a freezer at -20°C . When cooled, the reaction mixture was transferred to a separatory funnel and the vial rinsed with 25 ml of distilled water. A few drops of concentrated fluorometric grade sulfuric acid was added and the solution was extracted with 5 ml of chloroform. The chloroform solution was evaporated to dryness on a hot water bath under a stream of nitrogen, and 3 ml of absolute ethanol was added to dissolve the residue. The ethanol solution was transferred to quartz cuvettes and the solution was scanned on the fluorometer.

After recording the emission and excitation spectra, the reaction mixture was transferred from the quartz cuvette to a 10 ml beaker. The solution was carefully evaporated to a volume of 0.5 ml and equal portions were placed on two silica gel G thin layer plates. After developing the chromatograms in a tank containing hexane:ethyl ether (4:1), the plates were allowed to air dry and then viewed under black light. The fluorescent derivative products on the plates were marked. The derivative products were scraped off one of the plates and eluted with

absolute ethanol. The ethanol solutions were scanned on the fluorescence and ultraviolet spectrophotometers, evaporated to dryness, and then redissolved in 0.2 ml of deuterated chloroform. From this solution, nuclear magnetic resonance, infrared, and mass spectra were obtained. For the NMR studies, the solution was placed in a micro sample bulb and scanned 64 times with the time averaging system. For the mass spectra data, part of the solution was injected into the mass spectrometer while the rest of the solution was placed on NaCl salt plates for the infrared studies. The other thin layer plate was developed with a 1% diazo blue reagent spray.

To determine whether some of the other common cannabinoids would interfere with the procedure, solutions of cannabinol and cannabidiol were subjected to the same method of derivative preparations as for THC. The resulting products were scanned on the fluorometer to determine their excitation and emission spectra.

Two blood samples were prepared by dividing a 6-ml sample of blood into two equal parts and then pipetting 1 μ l of a 0.08 mg/ml Δ^9 -THC in ethanol solution into one portion. By following the extraction procedure, the Δ^9 -THC was extracted from the blood, placed in 1 ml of absolute ethanol, and then scanned on the fluorometer. After transferring to a reaction mini-vial, the Δ^9 -THC solution extract was carried through the derivatization procedure and then scanned on the fluorometer. The other 3-ml portion of blood was carried as such through the entire extraction and derivatization procedure as the blank.

Another reaction procedure was investigated by substituting sulfuric acid for the ethyl acetoacetate. In these experiments, 1 ml of

concentrated sulfuric acid was slowly added to each of several 3-ml mini-vials containing 1 ml of a solution of 100 $\mu\text{g/ml}$ of Δ^9 -THC in ethanol. The mini-vials were cooled in an ice water bath while adding the sulfuric acid. The vials were sealed with a screw cap, shaken to mix the solution, unscrewed to release the pressure, and then resealed. Then the vials were submerged in water baths ranging in temperatures from 55°C to 100°C for periods up to 18 hours. The reaction mixtures were transferred from the water baths to a freezer set to -20°C. When cooled, each reaction mixture was extracted with 5 ml of chloroform, evaporated to dryness on a hot water bath under a stream of nitrogen, and then redissolved with the addition of 3 ml of absolute ethanol. The solutions were transferred to quartz cuvettes and then scanned on the fluorometer. Cannabinol and cannabidiol were subjected to the same procedure.

Because both of these reaction procedures gave interfering fluorescence in the blanks, the fluorometer was modified by attaching a high pressure liquid chromatograph to an ultra-micro flow cell compartment replacing the standard cell compartment. A solution of 100 $\mu\text{g/ml}$ of Δ^9 -THC in cyclohexane was injected onto the liquid chromatograph Partisil 20 column using a cyclohexane:diozane (9:1) solvent system with a flow rate of 60 ml/hr. The fluorometer emission and excitation monochromators were set at 306 nm and 282 nm, respectively. The pen deflection on the recorder to the fluorometer was monitored for a period of 10 minutes.

CHAPTER IV

RESULTS AND DISCUSSION

Stability

During the initial part of the research, it was observed that there was a continuous decrease in the concentration of the stored Δ^9 -THC standard. Other investigators in the field of cannabinoid research have observed that the degradation product of Δ^9 -THC in its pure form is a violet-brown compound which rapidly appears on storage in the presence of oxygen (1). This compound is thought to be a polymer of Δ^9 -THC. In order to reduce this rate of decomposition it was necessary that both light and oxygen be prevented from influencing the standards. The problem of light was easily solved by using silanized amber glassware and by storing the solutions in darkness. Eliminating, or at least reducing, the influence of oxygen on THC was a more difficult problem. Two different approaches were tried. The first method was to introduce such additives as sodium nitrite, titanium trichloride, and ascorbic acid which might be oxidized by the oxygen present before the Δ^9 -THC would be attacked. Unfortunately these agents were found to alter the Δ^9 -THC more rapidly than did the oxygen. The second method was to exclude all traces of oxygen from the standard solutions. By applying a special handling technique in which oxygen was removed from both the ethanol solvent and the storage container, the decomposition rate was reduced considerably. Figure 3 presents the results of these tests.

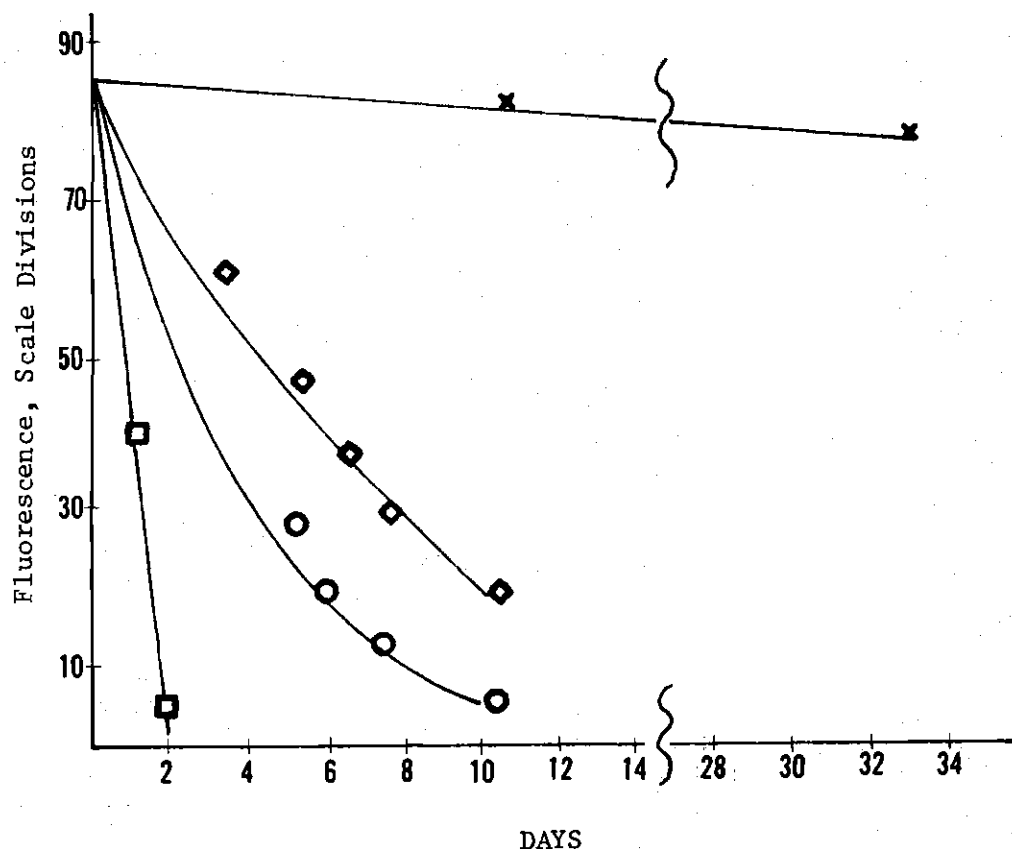


FIGURE 3. Stability Data: ♦ - Δ^9 -THC; ■ - NaNO_3 , TiCl_3 ; ● - Ascorbic acid; x - Δ^9 -THC purged EtOH

For the remainder of the research, all standard solutions of Δ^8 -THC and Δ^9 -THC were prepared by using nitrogen purged absolute ethanol, placed in silanized amber glass flasks which were purged with oxygen-free nitrogen, and stored in a freezer at -20°C . This method of storage reduced the rate of oxidative degradation of Δ^9 -THC to cannabinol, reduced the rate of conversion of Δ^9 -THC to Δ^8 -THC, and decreased the rate of polymer formation. It was also found possible to use these

storage methods to maintain standard solutions of cannabinol, cannabidiol, cannabigerol, cannabicyclol, and cannabichromene for a reasonable period of time.

Spectrophotometry

Absorption spectra of the various standard solutions were obtained utilizing the ultraviolet spectrophotometer. The solutions were scanned from 220 nm to 340 nm using the %T mode of the instrument (Appendix A). The two compounds of the greatest interest in this research, Δ^8 -THC and Δ^9 -THC, produced very similar spectra. Both compounds showed the typical absorption near 220 nm due in part to the $n \rightarrow \sigma^*$ antibonding orbital transitions of oxygen and in part to the hydroxyl substituted benzene (phenolic) portion of the tetrahydrocannabinol molecule. One would expect to see a very pronounced shift and a greatly intensified absorption in the secondary bands for benzene when substituted with a hydroxyl group. The hydroxyl group is a known auxochrome and when attached to a chromophoric system will usually cause a shift in the absorption to longer wavelengths (bathochromic shift) and a corresponding increase in the intensity of the absorption (hyperchromic effect). These were confirmed by the double bands at 276 nm and 282 nm. Transitions involving the nonbonding electrons were responsible for these effects. There was also noted a very close similarity between the secondary bands for Δ^9 -THC and of those for cannabidiol; however, the primary bands were different. The only difference between the spectra of Δ^9 -THC and Δ^8 -THC was in the intensity of the secondary band at 282 nm. This can be explained by the influence of the double bond at the Δ^9 position on the ring structure.

Δ^8 -Tetrahydrocannabinol at a concentration of 100 $\mu\text{g/ml}$ was easily detected with the ultraviolet spectrophotometer; however, a 1 $\mu\text{g/ml}$ solution produced no detectable signal. The E1% for Δ^8 -THC and for Δ^9 -THC were calculated as 36.4 and 55, respectively. The molar extinction coefficient, ϵ , was calculated as 1145 for Δ^8 -THC and 1729 for Δ^9 -THC (Appendix B). The respective values as reported by Ganoni (1) are 1320 and 1560.

The native fluorescence of each of the standard solutions was recorded (Appendix A). The maximum excitation energy at 282 nm is what was expected since the absorption maximum is at 282 nm. When both Δ^9 -THC and Δ^8 -THC were excited at 282 nm, the fluorescence maximum was recorded at 306 nm (Figure 4). The tetrahydrocannabinols process both the

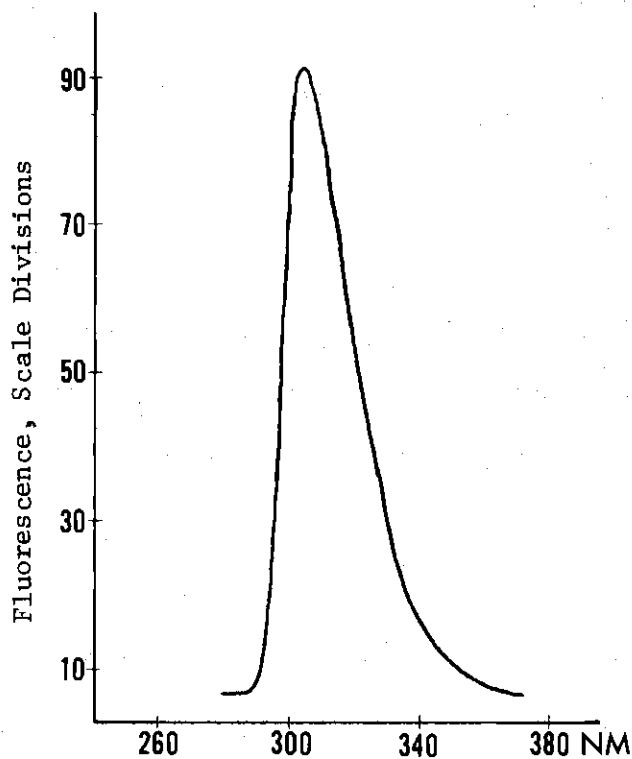


FIGURE 4. Fluorescence Spectrum of THC

molecular planarity and rigidity essential for maximum fluorescence. Chemical substitutions will normally only alter the fluorescence characteristics of a compound if the group is attached to the chromophoric system in a position such that it can produce perturbations in the mobility of the π electrons responsible for the fluorescence. This is what is observed in the tetrahydrocannabinol molecule. The benzene ring is substituted with a hydroxyl group giving rise to the presence of a phenolic system. The hydroxyl group is known as an ortho/para direction group and it will generally enhance fluorescence. The maximum emission at 306 nm when excited at 282 nm is what would be expected in as much as the phenolic system has an absorption maximum at 287 nm with a corresponding fluorescence at 310 nm.

It is known that a relatively small change in pH will sometimes radically affect the intensity and spectral characteristics of fluorescence. Most phenols are fluorescent in neutral or acidic media but the presence of a base leads to the formation of the non-fluorescent phenolate ion. Thus a pH of 1 will give maximum fluorescence for phenol while at a pH of 13 there is zero fluorescence. The dependence on pH was determined by the addition of acid and base to ethanolic solutions containing tetrahydrocannabinol. The addition of acid did not significantly affect the fluorescence intensity, there being noted only an approximate 5% decrease in the measured intensity. However, the addition of one drop of concentrated base decreased the fluorescence intensity to near zero.

Based upon the emission and excitation wavelengths required for maximum fluorescence and the effect of pH changes on the intensity of

that fluorescence, it appears obvious that the part of the tetrahydrocannabinol molecule most responsible for fluorescence is the hydroxyl substituted benzene ring, i.e. the phenolic moiety.

The fluorescence spectra of Δ^9 -THC in solutions of methanol, hexane, heptane, and isoamyl alcohol produced results similar to those in ethanol.

The quantum efficiency of Δ^8 -THC was calculated by using equation (I) to be 0.06 (uncorrected). The calculations are described in more detail in Appendix C. This is a reasonable value since Δ^8 -THC is a weakly fluorescent compound in comparison to quinine. This method of calculating the quantum yield is based on the relationship between the intensity of the emitted fluorescence F , the exciting intensity I_o , and the fluorescence efficiency ϕ , where

$$F = I_o \phi \quad (2.3 \text{ ecd}) \quad (\text{II})$$

When the fluorescence intensities of the two solutions are measured in the same fluorometer, using the same exciting source, at their respective maxima, then the two intensities are related by equation (I):

$$\frac{F_2}{F_1} = \frac{(\phi_2 \cdot A_2)}{(\phi_1 \cdot A_1)} \quad (\text{I})$$

The fluorescence intensity, F , is the entire emission representing the whole spectral output. For the present purposes, the area under the fluorescence spectrum can be used as F . Only if the absorption and fluorescence maxima of the two substances coincide can simple

calculations be made without corrections. In the present case, the absorption maxima for quinine and tetrahydrocannabinol are at different wavelengths and thus 0.06 is an uncorrected value.

Prolonged irradiation on Δ^9 -THC yielded one or more degradation products having emission maxima at 366 nm and 386 nm with an accompanying decrease in the fluorescence of the 306 nm peak. These emission peaks are probably due to cannabinol. The results are shown in Figure 5. The photodecomposition could be reduced by choosing another excitation wavelength or by reducing the excitation intensity via narrower slit widths or employing a neutral density filter.

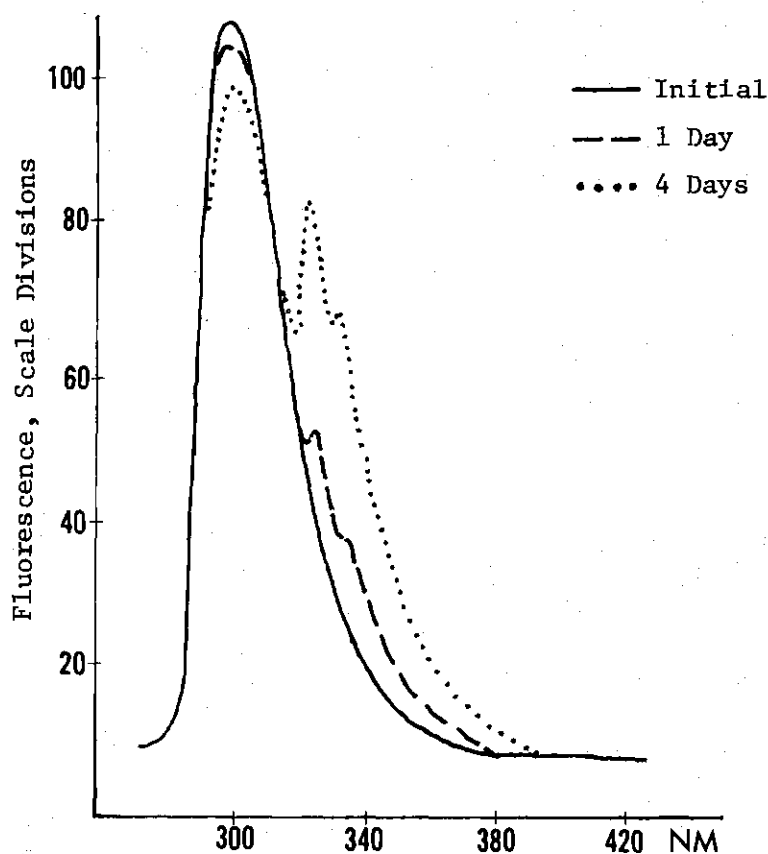


FIGURE 5. Decomposition of Δ^9 -THC Upon Irradiation

The effects of temperature on the fluorescence of Δ^9 -THC were determined by three experiments. In the first experiment the emission spectrum recorded at -1°C was compared to the spectrum obtained from the same solution at 25°C . The result was that a 46.5% increase in intensity was observed at the -1°C recording. In the second experiment, the solution was subjected to a temperature of -53°C . The intensity of the fluorescence at 306 nm was taken at 5 minute intervals until ambient temperature was reached. A 75° change in temperature resulted in over a 340% increase in the fluorescence (Figure 6). Since changes in temperature affect the viscosity of the medium and hence the number of collisions of the molecules of the fluorophore with solvent molecules, samples at lower temperature will generally produce a more intense emission

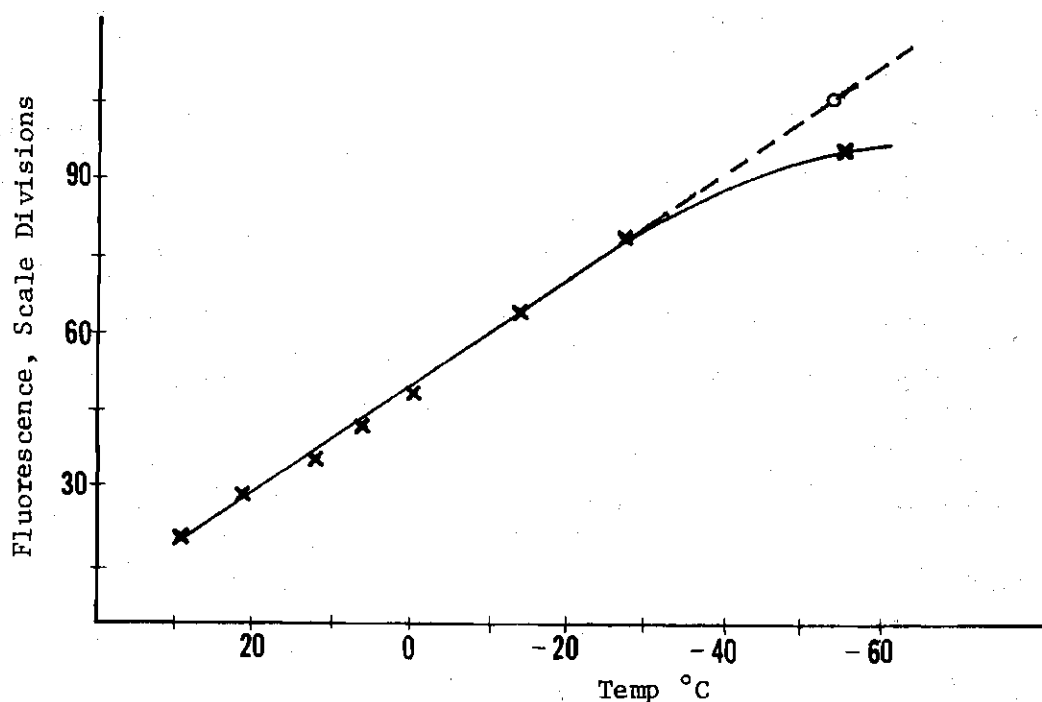


FIGURE 6. Temperature Dependence in CO_2 /Acetone Coolant

o = Least-squares regression

spectrum. It was expected that the intensity would level off when the solvent containing the THC became a solid. In fact, the results indicate that the ratio of the fluorescence intensity is a linear function of the temperature until approximately -40°C when a slight leveling trend was observed. In the final experiment liquid nitrogen (-193°C) was used to freeze solid the ethanol solvent containing Δ^9 -THC. The amount of motion of the THC molecules in the solvent obviously was drastically reduced. From Figure 7 it can be noticed that there is very little increase in fluorescence below -70°C . In conclusion, for cold temperature studies there appears to be no need to operate at temperatures below -80°C and at -50°C only a slight change ($<2\%$) would be noted in the fluorescence.

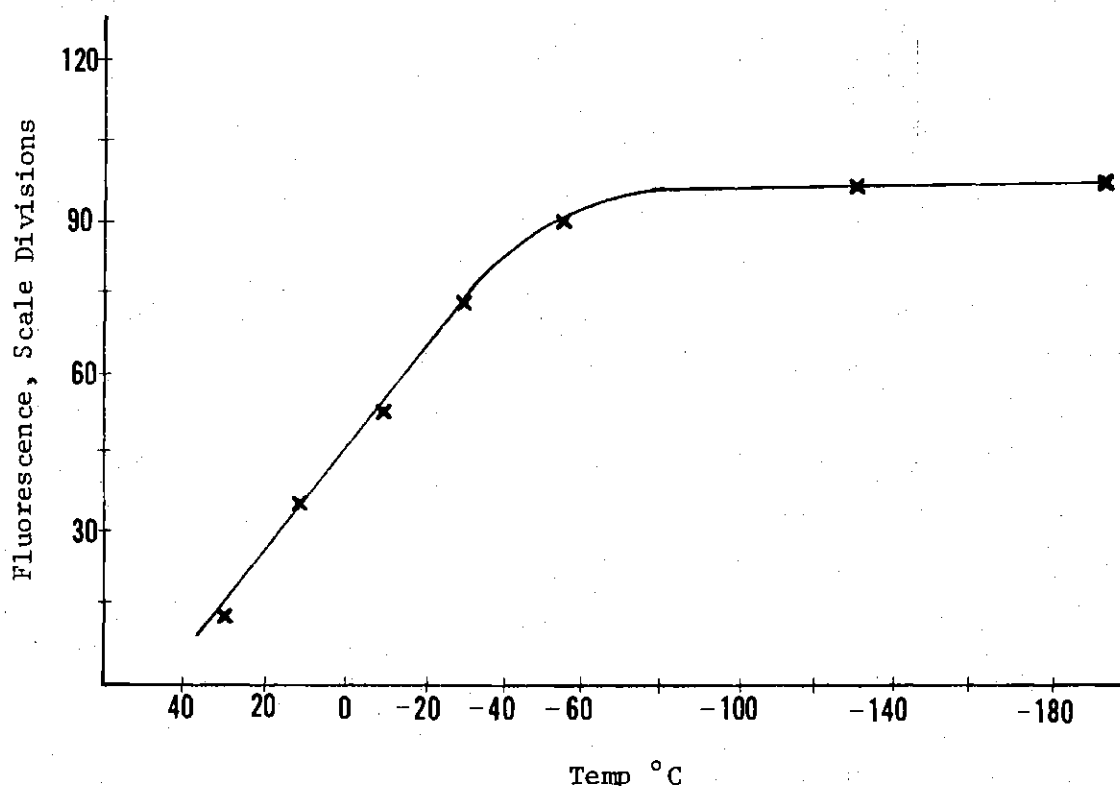


FIGURE 7. Temperature Dependence in Liquid Nitrogen Coolant

Extraction Procedures

The problem of isolating the cannabinoids from biological samples has plagued investigators for many years. Extraction methods must be able to remove both the protein-bound drug and the drug stored in the fat cells as well as the free drug. The major cannabinoids and their metabolites that are present in blood after smoking marijuana are Δ^8 -THC, Δ^9 -THC, cannabinal (CBN), 7-hydroxy- Δ^8 -THC, 6,7-dihydroxy- Δ^8 -THC, 7-hydroxy- Δ^9 -THC, 5,7-dihydroxy- Δ^9 -THC, and 7-hydroxy-cannabinal (23). Organic solvents such as hexane extracts the unchanged Δ^8 -THC and Δ^9 -THC, ether extracts the slightly more polar metabolites, while ethyl acetate extracts both. It would seem, then, that organic hydrocarbon solvents such as hexane would be the best choice to remove selectively the tetrahydrocannabinols while leaving the more polar metabolites in the blood. However, solvents immiscible with water can extract only a small fraction of the blood lipids because they are unable to cause more than a minimal disruption of the lipo-protein complexes. The cannabinoids fall into this class of lipid-soluble compounds. To bring about a simultaneous disruption of the complexes and extraction of the drug from plasma, it is necessary, therefore, to combine a water-soluble solvent with the water immiscible solvent. Various combinations of solvents such as chloroform-methanol, ether-ethanol, ether-isopropanol, and hexane-isoamyl alcohol have been reported in the literature. The n-heptane/isoamyl alcohol mixture as recommended by Lemberger (19) was reported to have a relatively high extraction efficiency and both of these latter solvents can be purchased as ultra pure, spectral grade chemicals.

After determining the EI% of Δ^8 -THC in n-heptane (Appendix C), several variations of the extraction procedure using n-heptane/isoamyl

alcohol were tested. An extraction efficiency of over 98% was obtained using the extraction procedure described in Chapter III. It was first tested on aqueous solutions and then on blood samples each containing known amounts of Δ^8 -THC. The solvent system gave equally satisfactory extraction efficiencies with these solutions.

The n-heptane/isoamyl alcohol mixture extracted the cannabinoids along with the other lipids present in the blood. By washing the collected organic layers with distilled water, the water soluble lipids were removed from the sample. Phenols that are insoluble in aqueous alkali can be extracted from organic solvents with Claisen's alkali. Since the tetrahydrocannabinols are insoluble in aqueous media, Claisen's alkali is an ideal extraction solvent for these phenolic compounds. Making the Claisen's alkali phase acidic converts the phenolate anions to the tetrahydrocannabinols which can be easily extracted with n-heptane.

Changing the n-heptane/isoamyl alcohol mixture from 98.5:1.5 to 95.5 did not affect the extraction efficiency. However, using only one 10-ml portion of n-heptane/isoamyl alcohol mixture did reduce the efficiency to an average of 75%. In conclusion, the extraction procedure developed was one in which a minimum number of steps were necessary to achieve a high extraction efficiency.

Derivatives of Tetrahydrocannabinols

Spectra grade solutions of methanol, hexane, heptane, isoamyl alcohol, and absolute ethanol were measured in the fluorometer at an excitation wavelength of 282 nm. At various instrumental sensitivity settings, the emission spectra scanned from 260 nm to 340 nm produced an array of fluorescent peaks. All of the above mentioned reagents had

impurities showing fluorescence in the region of 305-316 nm. This is probably due to trace amounts of acetone and other ketones present in the stock solutions as well as plasticizers that have contaminated the solvents during storage and transportation. Because of these impurities only a limit of 1 $\mu\text{g/ml}$ of THC was detectable. On the fluorometer used in this research, an additional sensitivity factor of 1000 would be available if the interfering impurities were absent.

Since the purpose of this research was to develop a practical fluorometric method for clinical or forensic laboratories, the purchasing of the required fluorometric grade reagents might become a prohibiting cost factor. Not many of the solvents are available in fluorometric grade and from experience in this research those that are available still may contain sufficient impurities so as to limit the level of detection for tetrahydrocannabinol.

It was considered that if a fluorescent derivative of tetrahydrocannabinol could be synthesized so as to effect a bathochromic shift in the emission spectrum, then many of the problems due to contamination might be solved. The von Pechmann-Duisberg synthesis of coumarins was chosen as a possible method because it seemed to offer the best chance of synthesizing a highly fluorescent derivative. Many phenols react very readily with β -keto esters in the presence of a dehydrating agent. To test this procedure, resorcinol was reacted with freshly distilled ethyl acetoacetate in the presence of fluorometric grade sulfuric acid. Sulfuric acid was chosen as the condensing agent because it is readily available in high purity and is also most effective in producing coumarins. However, with ethyl acetoacetate as the β -keto ester, a

mixture of a coumarin and a chromone may form in which the coumarin predominates.

Since the reaction with resorcinol was a success, a reaction was tried with tetrahydrocannabinol having substituted for resorcinol. This reaction generated no detectable fluorescent compounds. In general, the von Pechmann-Duisberg reaction mixtures need to stand overnight or for a number of days depending on the reactivities of the phenolic group involved and the β -keto ester used. Sometimes heat from a steam bath is required but the yield is generally low since a portion of the product is sulfonated. By applying heat from a hot water bath or working with an autoclave, a fluorescent compound was obtained when THC was reacted with ethyl acetoacetate in sulfuric acid.

The sulfuric acid was added gradually to the mixture of THC, ethanol, and ethyl acetoacetate with cooling since sufficient heat evolved may cause decomposition of the reactants or products.

When the reaction mixture was excited at 348 nm, an emission mixture at about 410 nm was recorded. It was determined that a reaction time of about 90 minutes on a steam bath at 90°C resulted in the greatest quantities of the fluorescent compounds.

In subsequent experiments, other dehydrating agents such as phosphorus pentoxide and phosphorus oxychloride were substituted for sulfuric acid. In general, phosphorus pentoxide causes the formation of a chromone instead of a coumarin. When this dehydrating agent was added to the reagent mixture, the initial reaction was vigorous and cooling was necessary. However, the reaction with phosphorus pentoxide did not product any favorable results either on a water bath at 60°C for one

hour or in an autoclave for 20 minutes at 20 PSI. In other instances phosphorus oxychloride has frequently been found to give better yields than sulfuric acid, but then dry benzene or toluene is usually the required solvent. The reaction mixture in such an attempt was heated for a few hours on a steam bath. A fluorescent product resulted with an emission peak at 403 nm when excited at 348 nm but the intensity was not as great as that of the original THC standard solution. Various autoclave and room temperature experiments using phosphorus oxychloride gave no compounds useful for fluorescence analysis.

From the results of the various experiments listed in Table I of Chapter III, the most successful procedure developed was the one in which tetrahydrocannabinol, ethyl acetoacetate, and sulfuric acid were reacted together for 90 minutes at 90°C. The blanks used in this procedure did not produce any significant fluorescent peaks at the desired wavelengths at a level equivalent to 2 ng/ml of Δ^9 -THC.

Having obtained a fluorescent compound that achieved the required bathochromic shift in the emission spectrum, the next step was to isolate the products from the reaction mixture. This was accomplished by using a well known thin layer chromatographic system for separating cannabinoids, i.e. hexane:ethyl ether (4:1). Unfortunately, not a single fluorescent derivative was evidenced by the black light but two intensely fluorescent bands occurred at Rf 0.20 and Rf 0.35, respectively. These bands suggest that possibly both a coumarin and a chromone were synthesized. After eluting the two bands with absolute ethanol, the solutions were scanned in the fluorometer. The top band gave an emission peak at 403 nm when excited at 343 nm; the respective data for the bottom band were

440 nm and 354 nm. During the course of the investigations to find the optimum experimental parameters, on several occasions a fluorescent peak at 440 nm instead of 403 nm was recorded. Numerous degradation products were observed along with unreacted Δ^9 -THC when the second thin layer plate was treated with a 1% diazo blue spray. From these results it can be concluded that the procedure did not achieve the required 100% conversion of THC to a single fluorescent derivative.

The absorption spectra of the derivatives are similar to that of tetrahydrocannabinol (Appendix A). A small bathochromic shift accompanied by a hyperchromic effect is noticeable in the absorption spectra. This suggests that a chromophore has been attached to the phenolic ring.

The infrared spectra of the derivatives are very similar to the infrared spectrum of Δ^9 -THC. The major differences are due to the addition of the carbonyl absorption at 1710 cm^{-1} . However, the carbonyl absorptions are weaker than expected, and with a broader OH band the possibility of enolization is a viable conclusion. There is also a noticeable decrease in the intensity of the OH bending absorption at 1430 cm^{-1} .

NMR spectra of the Δ^9 -THC and of the derivative are rather complex and make interpretation very difficult. All of the spectra have the doublet at approximately 6.2 δ and 6.7 δ which is attributed to the two hydrogens on the benzene ring. From this data it can be concluded that there has been no ring closure as expected at the Δ -2 position. Apparently, the steric hindrance caused by the alkane chain at the ortho position is great enough to restrict the von Pechmann-Duisberg reaction. The singlet at 4.9 δ on the standard Δ^9 -THC NMR spectrum which corresponds

to the hydrogen from the hydroxyl group is absent in the derivative spectra. The lack of this signal indicates that the first step of the reaction was successful in attaching part of the ethyl acetoacetate to the THC molecule.

The mass spectra of the derivative products present a fragmentation pattern up to 314 mass units which is nearly identical to the fragmentation pattern of Δ^9 -THC. The apparent molecular ion (parent) peak of 398 is consistent with attaching $\text{COCH}_2\text{COCH}_3$ to the phenolic ring. The prominent peaks at 381, 341, 314, and 313 can be explained by simple cleavages from the β -diketone group from the compound.

In conclusion, the spectral data are in good agreement with the formation of a β -diketone derivative of tetrahydrocannabinol at the Δ -1 position. Even though neither a coumarin nor a chromone was synthesized, the derivative compounds generated were highly fluorescent.

Cannabinol and cannabidiol were subjected to the von Pechmann-Duisberg derivative procedure. These two cannabinoids were selected because in comparison to the other cannabinoids they are present in a relatively high proportion in marijuana. From the results of these experiments, it was determined that cannabinol did not interfere but that cannabidiol gave results very similar as to those obtained with tetrahydrocannabinol. Cannabidiol was the most likely compound to interfere since for the reaction to occur it has two active sites on the benzene portion of the molecule.

In the experiments in which blood was substituted for the aqueous solutions described earlier, apparently the same fluorescent compounds were obtained. Unfortunately, the blood blanks produced a fluorescent signal equivalent to 60% of that from the 80 $\mu\text{g/ml}$ of Δ^9 -THC solution.

After the extraction procedure was performed, the blood blanks produced a fluorescence at about 400 nm when excited at 345 nm. This indicates that blood contains fluorescent compounds that are not removed by the extraction procedure. Blood is known to contain an enormous amount of trace foreign substances, among these acetone and various types of phenolic compounds.

Two approaches to the problem due to these impurities were attempted. The first one was to find a reaction procedure that would produce a fluorescent compound without generating interfering fluorescence from trace materials present in blood. The procedure investigated was one in which 1 ml of concentrated sulfuric acid was substituted for the 1 ml of 5% ethyl acetoacetate solution used in the previous derivatization experiment. This experiment is similar to one that was proposed by Dal Cortivo (21) in which morphine is converted into a fluorophor. By the addition of concentrated sulfuric acid to morphine, a highly fluorescent dimer is formed as some investigators believe. This idea was tested by reacting Δ^9 -THC with concentrated sulfuric acid submerged in a water bath for a period of time. A fluorescent compound was obtained that had an emission at 480 nm when excited at 420 nm. To find the optimum experimental conditions, temperature and time were varied (Figure 8). The reaction temperature of 70°C and reaction time of 90 minutes were necessary for product formation. Both cannabiol and cannabidiol gave similar fluorescence spectra when subjected to the same experimental conditions as THC. This procedure lacked selectivity since it seemed that the dimers of the various cannabinoids that were generated had very similar fluorescent properties.

The second approach was to remove those substances and other cannabinoids that caused the interference. If Δ^9 -THC could be separated from these undesirable compounds, then both the native fluorescence as well as the derivative fluorescence could be measured. To separate THC from those compounds present in blood that could have caused the undesirable fluorescence, a high pressure liquid chromatograph (HPLC) was interfaced to the fluorometer via an ultra-micro flow cell. In effect, the fluorometer became a highly sensitive fluorescence detector for the HPLC. However, the scattered light due to the design of the cylindrical flow cell reduced the effectiveness of the system to only a 50 ng/ml detection limit. This was not low enough for the purpose of this research. A new rectangular cell has become commercially available for the ultra-micro flow cell system, and with it a lower detection limit might be possible. Due to the fact that this rectangular cell could not become available for this research before the manuscript was completed, this system was not tested.

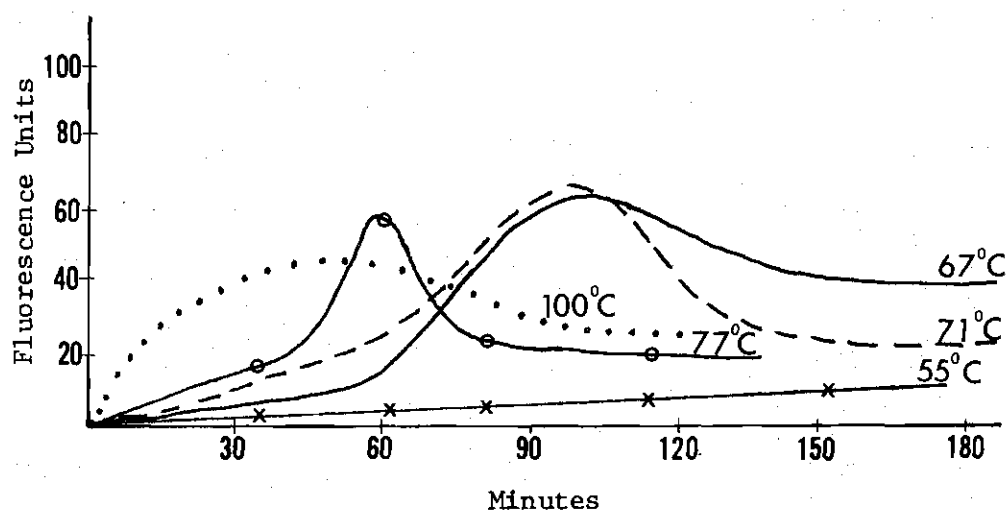


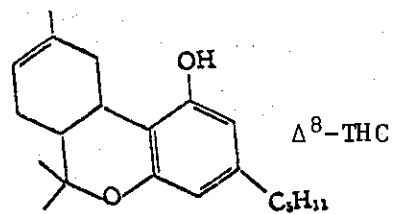
FIGURE 8. Temperature and Time Dependence in Sulfuric Acid/THC Reaction

CONCLUSIONS

The tetrahydrocannabinols can be determined by fluorometry if sufficient care is taken to remove interfering compounds. The tetrahydrocannabinols can be efficiently extracted from blood samples. The fluorescent derivatives of Δ^9 -THC generated by the von Pechmann-Duisberg reaction and by the Dal Cortivo reaction lacked both the selectivity and the sensitivity required for the determination of cannabis intoxication.

The technique of interfacing the high pressure liquid chromatograph to the fluorometer with an ultra-micro flow cell could be further modified by replacing the cylindrical cell with the recently developed rectangular cell. By using this system it might be possible to separate the tetrahydrocannabinols from the interfering fluorescent compounds present in blood. Thus further investigations into other fluorescent derivatives of tetrahydrocannabinol would seem to be warranted.

APPENDIX A



Absorbance ($\lambda_{\text{max}}^{\text{EtOH}}$): 282 nm, 278 nm,
225 nm

Fluorescence: ex 282 nm, em 306 nm

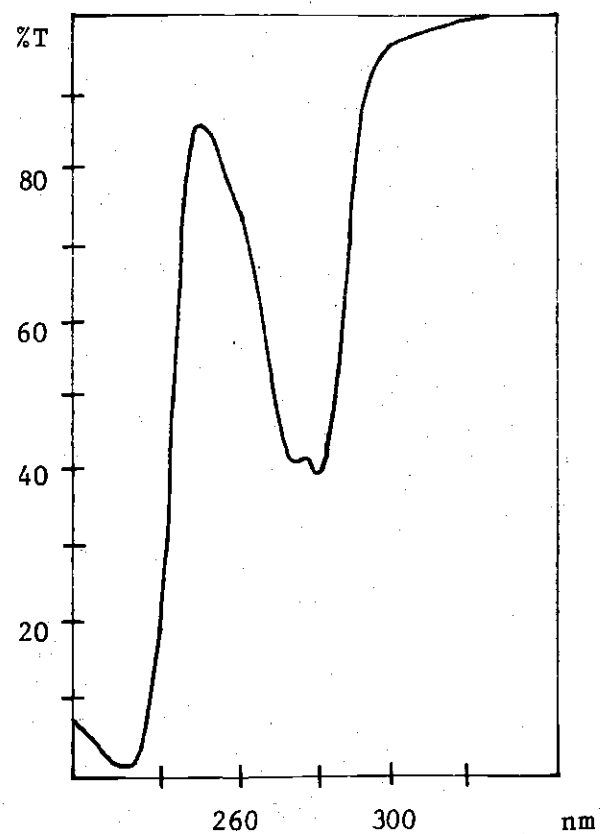
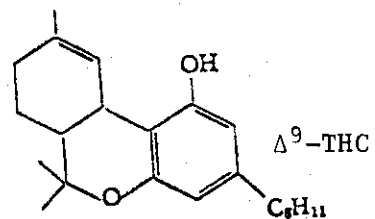


FIGURE 9. Spectrophotometric Data for Δ^8 -Tetrahydrocannabinol



Absorbance ($\lambda_{\text{max}}^{\text{EtOH}}$): 282 nm, 278 nm,
225 nm

Fluorescence: ex 282 nm, em 306 nm

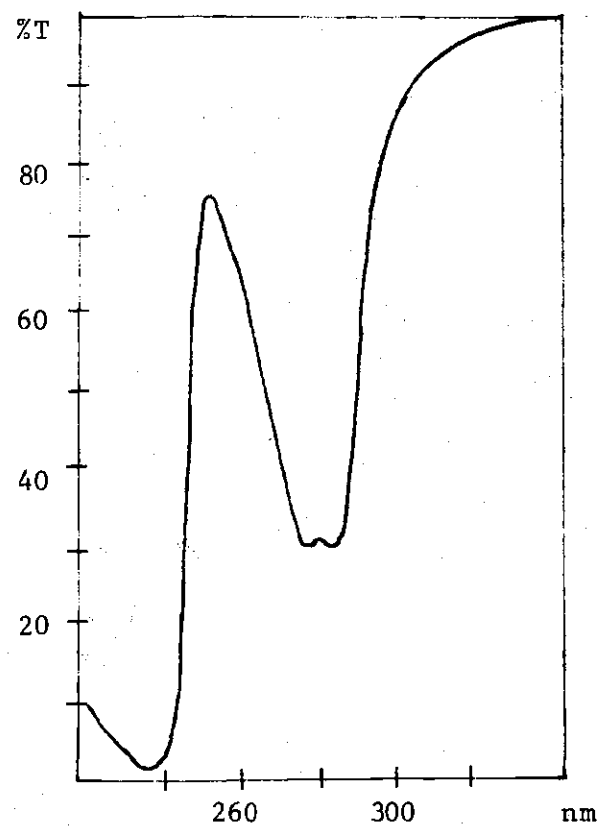
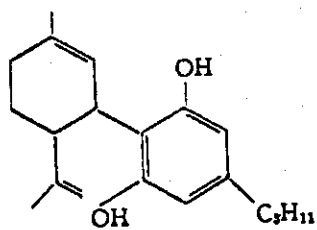


FIGURE 10. Spectrophotometric Data for Δ^9 -Tetrahydrocannabinol



Absorbance ($\lambda_{\text{max}}^{\text{EtOH}}$): 282 nm, 274 nm

Fluorescence: ex 283 nm, em 306 nm

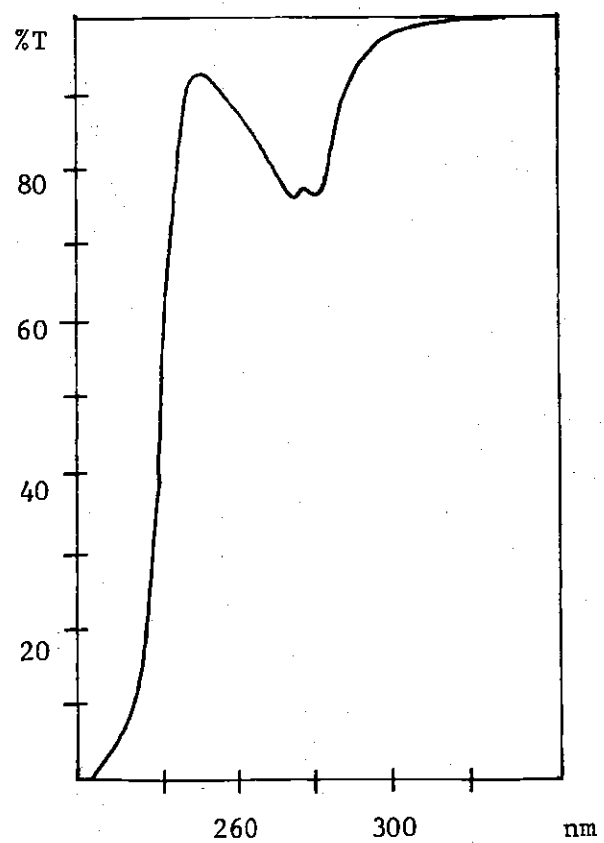
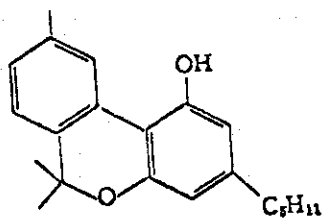


FIGURE 11. Spectrophotometric Data for Cannabidiol



Absorbance ($\lambda_{\text{max}}^{\text{EtOH}}$): 286 nm

Fluorescence: ex 288 nm, em 360 nm
and 380 nm

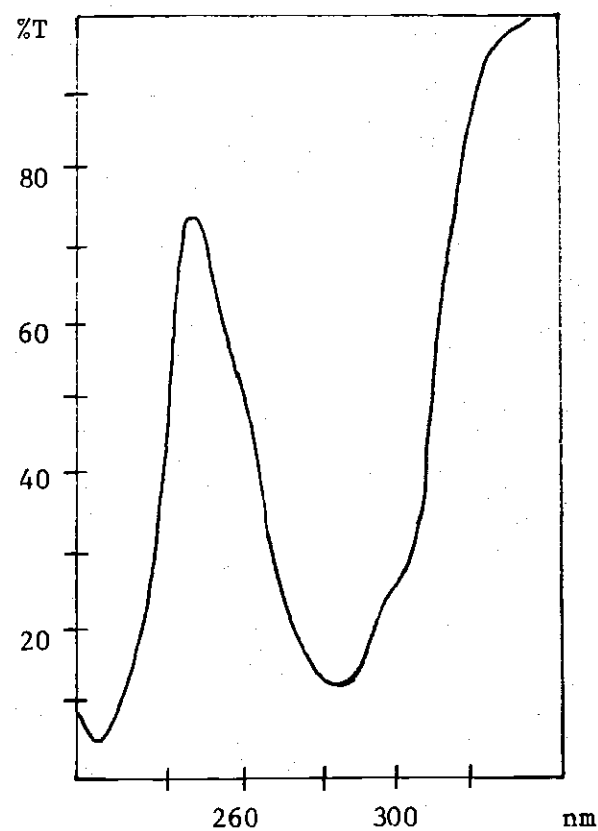
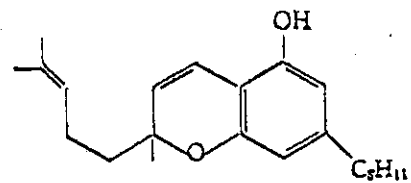


FIGURE 12. Spectrophotometric Data for Cannabinol



Absorbance ($\lambda_{\text{max}}^{\text{EtOH}}$): 280 nm, 286 nm

Fluorescence: none

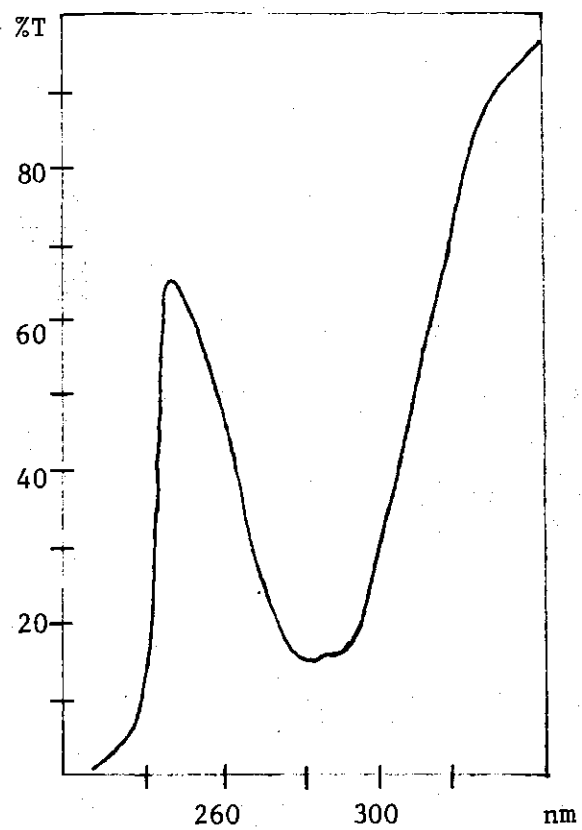
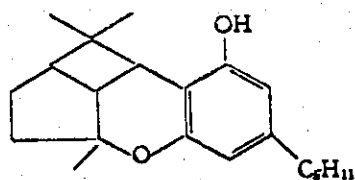


FIGURE 13. Spectrophotometric Data for Cannabichromene



Absorbance ($\lambda_{\text{max}}^{\text{EtOH}}$): 282 nm, 274 nm

Fluorescence: ex 280 nm, em 300 nm

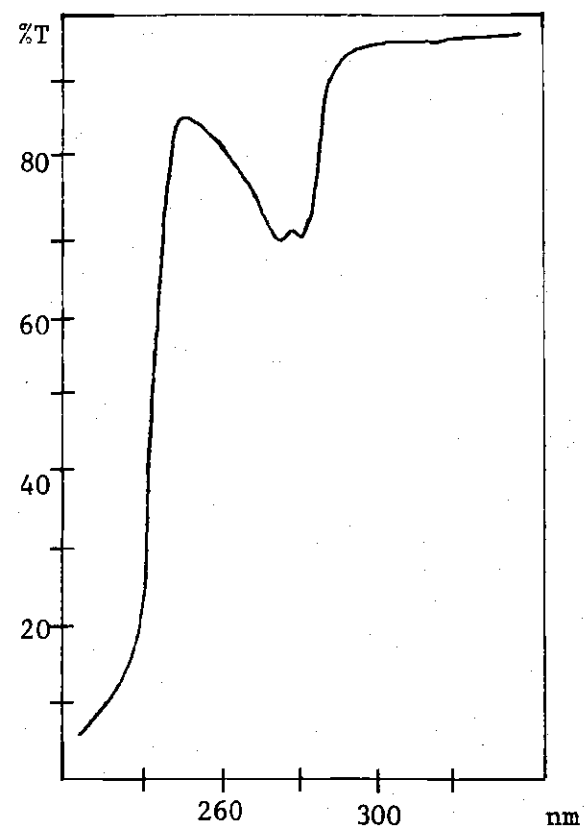
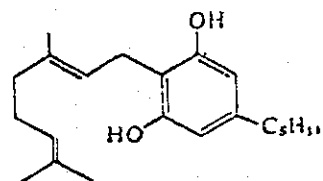


FIGURE 14. Spectrophotometric Data for Cannabicyclol



Absorbance ($\lambda_{\text{max}}^{\text{EtOH}}$): 280 nm, 270 nm

Fluorescence: ex 277 nm, em 299 nm

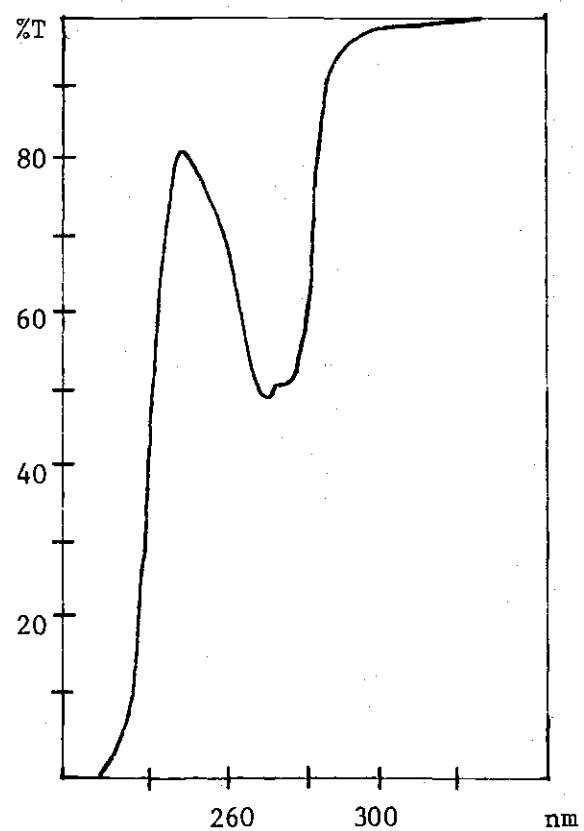
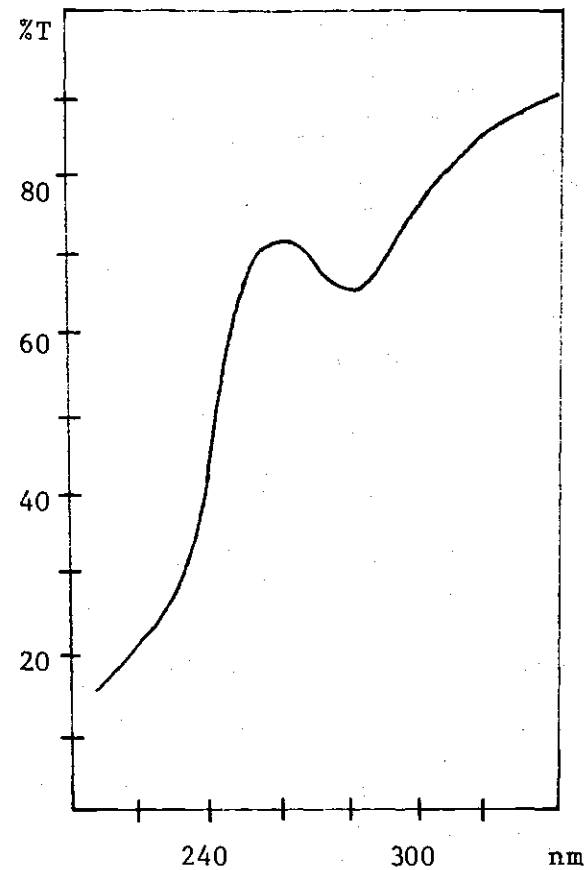
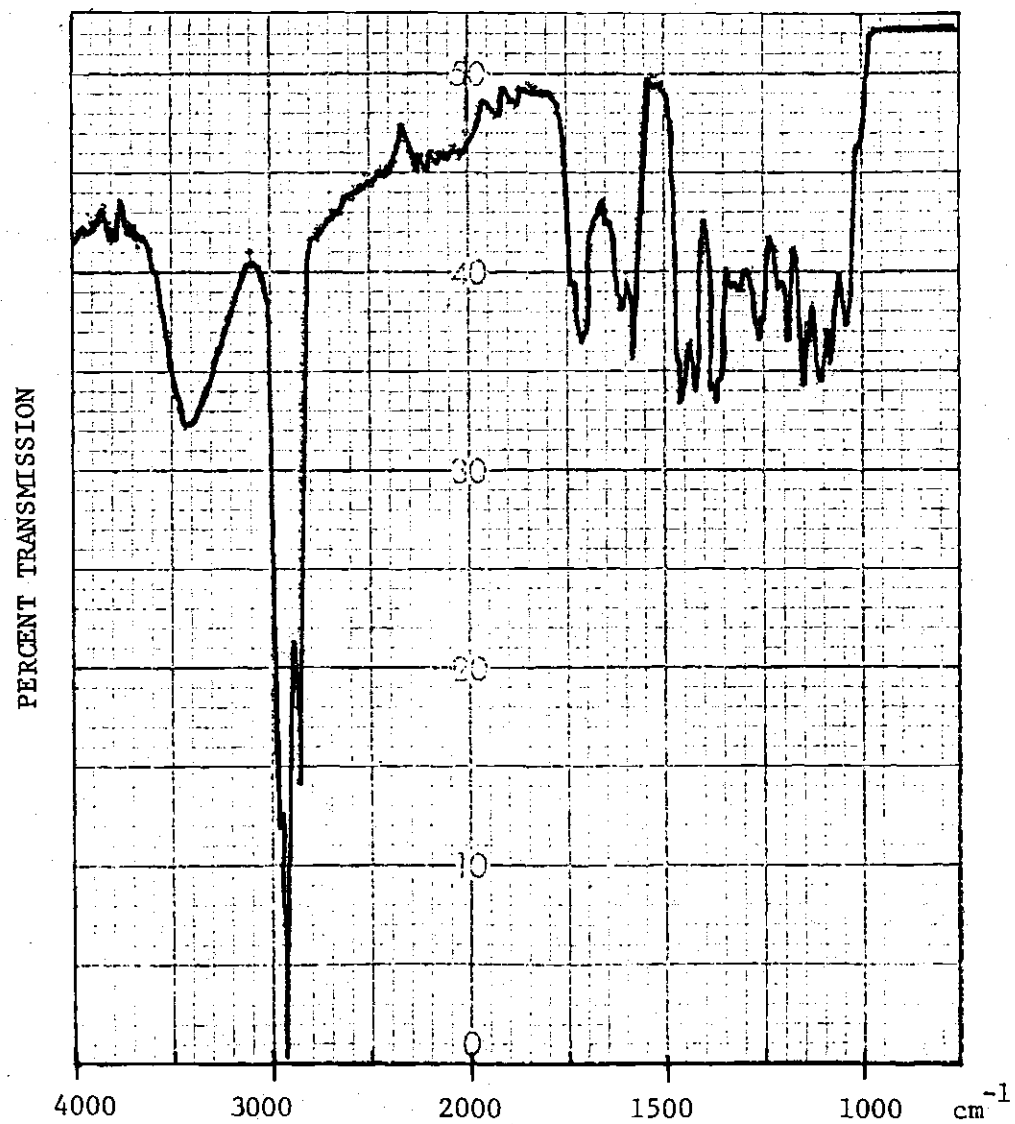
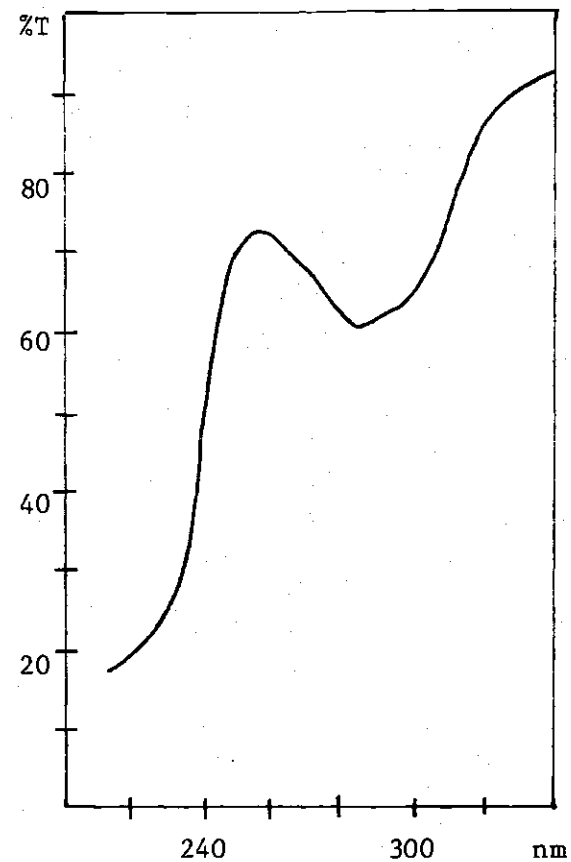
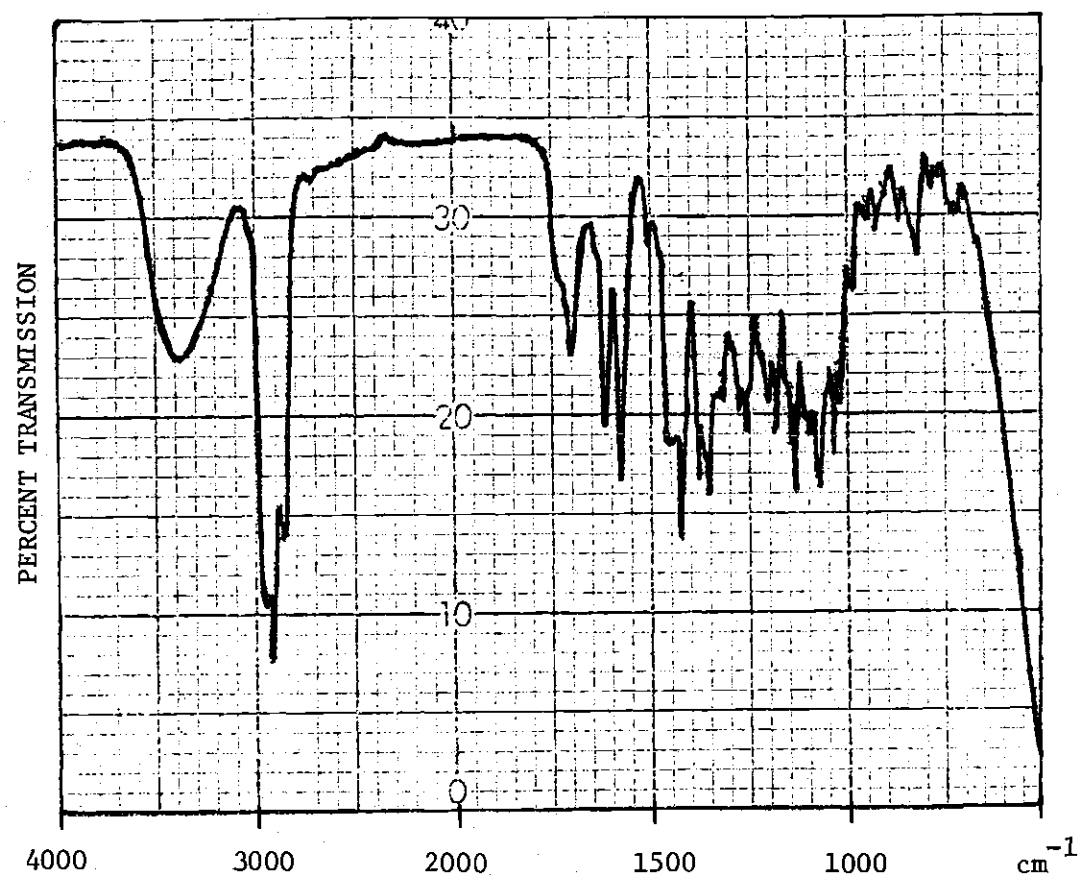


FIGURE 15. Spectrophotometric Data for Cannabigerol



Absorbance ($\lambda_{\text{max}}^{\text{EtOH}}$): 280 nm

FIGURE 16. Infrared and Ultraviolet Spectra for Compound Labeled "Top Band"



Absorbance ($\lambda_{\text{max}}^{\text{EtOH}}$): 282 nm, 300 nm

FIGURE 17. Infrared and Ultraviolet Spectra for Compound Labeled "Bottom Band"

APPENDIX B

E1% AND MOLAR ABSORPTIVITY CALCULATIONS

The E1% for Δ^8 -THC and Δ^9 -THC in ethanol can be calculated by using equation

$$E1\% = (A \cdot 10) / c, \quad (III)$$

where A is the absorbance and c is the concentration in gm/l. In the ultraviolet spectrophotometer at a wavelength setting of 282 nm, a solution containing 100 μ g/ml of Δ^8 -THC in absolute ethanol gave a %T reading of 43.4. Converting 43.4 %T to A, one obtains a value of 0.364 A units. By substituting the values of A and c into equation (III), one obtains a value of 36.4 l/gm-cm for the E1% of Δ^8 -THC.

The same procedure was repeated for an 80 μ g/ml solution of Δ^9 -THC in absolute ethanol. A value of 0.44 A units gave a value of 55 l/gm-cm for the E1% of Δ^9 -THC.

A 50 μ g/ml of Δ^8 -THC in n-heptane was scanned on the ultraviolet spectrophotometer and a value of 0.174 A units was recorded. The E1% of Δ^8 -THC in n-heptane was calculated using equation (III) to yield a result of 34.8 l/gm-cm.

The molar absorptivity can be calculated using equation

$$\epsilon = (E1\% \cdot \text{Mol. wt.}) / 10 \quad (IV)$$

where the molecular weight of THC is 314.5 atomic mass units. Substituting the E1% and molecular weight into equation (IV), the ϵ is calculated to be 1145 liters/mole-centimeter for Δ^8 -THC and 1729 liters/mole-centimeter for Δ^9 -THC.

APPENDIX C

QUANTUM EFFICIENCY

By rearranging equation

$$\frac{F_{\text{THC}}}{F_{\text{Quinine}}} = \frac{(\phi_{\text{THC}} \cdot A_{\text{THC}})}{(\phi_{\text{Quinine}} \cdot A_{\text{Quinine}})}, \quad (\text{I})$$

one obtains equation (V).

$$\phi_{\text{THC}} = (F_{\text{THC}} \cdot A_{\text{Quinine}} \cdot \phi_{\text{Quinine}}) / (F_{\text{Quinine}} \cdot A_{\text{THC}}) \quad (\text{V})$$

The fluorescence intensity, F , can be calculated in terms of the weight of the spectrum's area. From the measurements recorded during the experiment and from the known quantum value of quinine, where

$$F_{\text{THC}} = 2.00$$

$$F_{\text{Quinine}} = 4.49$$

$$A_{\text{THC}} = 0.364$$

$$A_{\text{Quinine}} = 0.089$$

$$\phi_{\text{Quinine}} = 0.55$$

the value of ϕ_{THC} is calculated as 0.06 (uncorrected). The absorbance values were obtained by scanning the solutions in the ultraviolet spectrophotometer after the fluorescence spectra were recorded.

BIBLIOGRAPHY

1. Mechoulam, R., ed., Marijuana: Chemistry, Pharmacology, Metabolism and Clinical Effects, Academic Press, New York, N.Y., (1973), pp. ziii, 149, 151.
2. Marijuana and Health, Fifth Annual Report to the U.S. Congress, (1975), p. 85.
3. Jones, H., Sensual Drugs, Cambridge University Press, Cambridge, Mass., (1977), p. 284.
4. Da Silva, J.B., Rev. Fac. Farm Bioquim, University Sao Paulo, 5, 205 (1967).
5. Just, W.W., Werner, G., and Wiechmann, M., Naturwissenschaften, 59, 222 (1972).
6. Forrest, I.S., et al., Res. Commun. Chem. Pathol Pharmacol, 2, 787 (1971).
7. Fenimore, D.C., Freeman, R.R., and Lay, P.R., Anal. Chem., 45, 2331 (1973).
8. Garrett, E.R., and Hunt, C.A., J. Pharm. Sci., 62, 1211 (1973).
9. McCallum, N.K., J. Chromatogr. Sci., 11, 509 (1973).
10. Agurell, S., et al., J. Pharm. Pharmacol., 25, 554 (1973).
11. Rosenfeld, J.H., et al., Anal. Chem., 46, 2232 (1974).
12. Mechoulam, R., McCallum, N.K., and Burstein, S., Chem. Rev., 76, 98 (1976).
13. Cais, M., et al., FEBS Letter, 55, 257 (1975).
14. Bowd, A., Byron, P., Hudson, J.B., and Turnbull, J.A., Talanta, 18, 697 (1971).
15. Vinson, J.A., Patel, D.D., and Patel, A.H., Anal. Chem., 49, 163 (1975).
16. Bourdon, R., Marijuana: Chemistry, Biochemistry, and Cellular Effects, Nahas, G.G., ed., Springer-Verlag, New York, N.Y., (1976), pp. 111-119.

17. Bullock, F.J., U.S. Patent 3,656,906, April 18, 1972.
18. Lemberger, L., et al., Sci., 170, 1320 (1970).
19. Sethna, S., and Phodke, P., Organic Reactions, Vol. VII, Adams, R., ed., John Wiley and Sons, Inc., New York, N.Y., (1953), pp. 1-58.
20. Pesez, M., and Bartos, J., Colorimetric and Fluorimetric Analysis of Organic Compounds and Drugs, Marcel Dekker, Inc., New York, N.Y., (1974), p. 109.
21. Dal Cortivo, L.A., "Fluorometric Determination of Morphine", paper at the 156th National Meeting of the American Chemical Society, N.Y., (1967).